

INVOLVEMENT OF MITOCHONDRIAL TRANSCRIPTION FACTOR (TFAM) IN
PORCINE GAMETOGENESIS AND PREIMPLANTATION
EMBRYO DEVELOPMENT

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

INVOLVEMENT OF MITOCHONDRIAL TRANSCRIPTION FACTOR A (TFAM) IN
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DEVELOPMENT

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ABSTRACT

INVOLVEMENT OF MITOCHONDRIAL TRANSCRIPTION FACTOR (TFAM) IN PORCINE GAMETOGENESIS AND PREIMPLANTATION EMBRYO DEVELOPMENT

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Mitochondrial Transcription Factor A (TFAM) is responsible for stability, maintenance, and transcriptional control of mitochondrial DNA (mtDNA) within the mitochondrial nucleoid. Heterozygous TFAM knockout mice exhibit depletion of mtDNA and a homozygous knock-out is embryo-lethal, implying that TFAM has an essential role in mitochondrial biogenesis and sustenance during preimplantation embryonic development. We have studied the expression and distribution of TFAM in the gametes and preimplantation embryos of the domestic pig (*Sus scrofa*), a livestock species of major agricultural and economic significance. We hypothesized that TFAM will be expressed at high levels in oocytes and embryos to support their development. We also anticipated that sperm mitochondria should be devoid of TFAM since they are destined for degradation after fertilization. The content of TFAM mRNA increased considerably during porcine oocyte maturation and preimplantation development of porcine embryos. TFAM protein accumulated in the cytoplasm of porcine oocytes during *in vitro* maturation and was reduced by proteolysis after fertilization. This pattern of TFAM accumulation was not mirrored by parthenogenetically activated oocytes and

zygotes reconstructed by somatic cell nuclear transfer (SCNT), suggesting deviant processing of TFAM protein and transcript after oocyte/embryo manipulation. The appropriate band of 25 kDa was detected by western blotting of both meiotically mature ova and zygotes, and was also present in ejaculated boar spermatozoa. Boar sperm extracts also displayed several bands of lesser mass, indicative of proteolytic degradation, and several bands >25 kDa suggestive of posttranslational modification by ubiquitination. Ubiquitination of sperm TFAM was confirmed by affinity purification of ubiquitinated proteins using matrix immobilized ubiquitin-binding protein p62, followed by TFAM western blotting. The accumulation of TFAM protein was observed in the cytoplasmic lobes of late stage elongating spermatids on boar testicular tissue sections. TFAM immunoreactivity was absent from the mitochondrial sheath, and relegated to the sperm tail principal piece and cytoplasmic droplet in fully differentiated spermatozoa. Isolation of sperm heads and sperm tails confirmed that TFAM protein is present in the principal piece of the sperm tail. These data suggest that mitochondrial transcription factor TFAM is developmentally regulated in porcine gametes and embryos, and may exert a critical role in gametogenesis and preimplantation embryo development. Sperm TFAM protein could be marked for proteolysis by the ubiquitin-proteasome pathway during spermatid elongation to facilitate the degradation of paternal mitochondria after fertilization.

CHAPTER I

INTRODUCTION

Mitochondrial diseases are among the most prevalent inherited diseases that affect one out of 7,634 newborns (Debray et al., 2007). Mitochondrial function affects virtually all body systems, including the reproductive system. In order to appreciate the biological significance of mitochondrial function and inheritance in gametogenesis and embryo development, we examined a major protein involved in the control of mitochondrial DNA sustenance and replication in the porcine gametes and preimplantation embryos. Mitochondrial transcription factor A (TFAM) plays a primary role in stability and maintenance of mtDNA (Fisher and Clayton, 1988) as well as regulation of mtDNA copy number (Scarpulla, 2006). Knockout mice lacking the *Tfam* gene do not survive past embryonic day 8.5 (Larsson et al., 1997a). Similar to other systems, TFAM may control mitochondrial copy number in gametes and embryos and promote the desired maternal mode of mtDNA inheritance after fertilization. We also investigated the regulation of TFAM in porcine embryos reconstructed by somatic cell nuclear transfer. Such important proposed roles of TFAM and mitochondrial function in the reproductive system of large livestock species are of critical importance to the agricultural and animal science community. Our main hypotheses are 1) TFAM is removed from the sperm mitochondria during porcine spermiogenesis to reduce the likelihood of propagation of paternal mitochondria and paternal mtDNA and 2) maternal TFAM is essential for porcine pre-implantation development.

CHAPTER II

LITERATURE REVIEW

The Mitochondrion

Mitochondria play an important role in cellular function and in numerous pathologies. Mitochondrial diseases are among the most prevalent inherited diseases estimated to affect one out of 7,634 newborns (Debray et al., 2007). Major diseases associated with mitochondrial dysfunction include Parkinson's disease, diabetes mellitus, Kearns-Sayre syndrome, Alzheimer's disease; and the aging process has also been associated with mitochondrial dysfunction (Scheffler 2001; Sanaker, Husebye et al. 2007). Mitochondrial positioning may create ATP and calcium gradients that are mandatory for normal embryo development (Schatten et al., 2005). Furthermore, mitochondria have an essential role in apoptosis which has strong implications for cancer research, developmental biology, and senescence and death (Scheffler, 2001).

The exact origin of mitochondria is not known, but many believe that mitochondria have evolved from endosymbionts of the primitive eukaryotes due to this organelle having marked properties of prokaryotes while functioning within an eukaryotic organism (Margulis, 1981). The *serial endosymbionts* theory suggests that a protoeukaryotic cell lacking mitochondria endocytosed a proteobacterium, a precursor of the mitochondrion, thus developing a symbiotic relationship. This process would eventually result in the loss of redundant proteobacterial genes and incorporation of

crucial respiratory chain genes from proteobacterium to the recipient cell nuclear genome (Gray, 1993; Gray et al., 1999). An alternative theory termed *revisionist view of eukaryotic evolution* implicates a fusion of anaerobic archaebacteria as the host and a respiration-competent proteobacteria as the symbionts that merge to form an eukaryote (Scheffler, 2001). Mitochondria are surprisingly unique since the mitochondria from one mammalian species typically do not function in the cytoplasm of a species with a different nuclear genome (Scheffler, 2001).

The first observations of mitochondria by electron microscope noted a basic morphology of a matrix enclosed by the inner mitochondrial membrane and the inner membrane space between the inner and outer mitochondrial membrane. The term *cristae* was coined for the folds of inner mitochondrial membrane, which add to the total membrane area of the mitochondrion (Scheffler, 2001). As advances in mitochondrial research were made, the continuous surface area of the inner membrane was divided into two domains, one called the inner boundary membrane due to its frequent contact with the outer mitochondrial membrane, and the other portion that forms the *cristae* with tubular or lamellar structures (Scheffler, 2001).

The maintenance of a sufficient pool of mitochondria during cell division and proliferation is assured by the process of fusion and fission (Bereiter-Hahn and Voth, 1994). The process of fission might be obvious as a parallel to the mechanism of cell division and multiplication but the state of mitochondrial fusion is less apparent. Perhaps proteins are constantly moved and exchanged between the mitochondria or moved within the mitochondrial reticulum. The mtDNA is more strictly regulated which prevents heteroplasmy during cell division and generational mtDNA segregation (Scheffler, 2001).

In yeast the mitochondrial reticulum maintenance is highly dependent on constant fusion and fission (Nunnari et al., 1997).

The primary function of mitochondria is in energy metabolism and generation of ATP, as well as in the Krebs's cycle and urea cycle. Fatty acid oxidation was also discovered as a function of this organelle (Scheffler, 2001). Steriodogenesis has been suggested to be a function of mitochondria as well, such as an involvement in testosterone synthesis in the Leydig cells in the testis (Manna et al., 1999). In the pig, steriodogenesis in non-conceptus tissue is regulated by steroidogenic acute regulatory protein (StAR) which is dependent on the transport of cholesterol across the mitochondria from the outer membrane to the inner membrane (Blomberg and Zuelke, 2005). This cholesterol transport is necessary in order for ovarian steriodogenesis to occur (Rusovici et al., 2005). Mitochondria also participate during the execution phase of apoptosis (Martin et al., 2007).

Cellular distribution and function of mitochondria is based on protein interactions located on the outer mitochondrial surface as well as on mitochondrial membrane interactions with the cytoskeletal elements such as actin filaments, intermediate filaments, and microtubules (Scheffler, 2001). Multiple mitochondrial proteins interact with the cytoskeleton-associated motor proteins can directly move mitochondria along microtubules or microfilaments (Scheffler, 2001). Microtubules partake in active translocations of cellular organelles and intracytoplasmic protein transport which is affected by the organization of microtubules during pig fertilization and early development (Schatten et al., 2005). The translocation of the mitochondria inside the fertilized egg is controlled by the zygotic centrosome and sperm aster microtubules

emanating from it. In all mammalian species except rodents, the sperm-derived paternal centriole plays a primary role in the reconstitution of the zygotic centrosome and nucleation of sperm aster microtubules, ultimately allowing the maternal and paternal genomes to combine during the fertilization process. The centrosome is also associated with the donor cell nucleus after SCNT. Abnormal microtubule organization in the zygote results in asymmetric mitochondrial distribution (Van Blerkom et al., 2000; Van Blerkom et al., 1995). Mitochondrial positioning by microtubules may help maintain ATP and calcium gradients necessary for normal development of the embryo (Schatten et al., 2005). Other factors that can affect mitochondrial function are changes in pH, differences in phosphorylation, interactions of centrosomal and nuclear proteins during transition from interphase to mitosis, and calcium (Schatten et al., 2005).

Mitochondrial DNA (mtDNA)

The mtDNA must be replicated and transcribed in order for mitochondrial multiplication to occur (Scheffler, 2001). Human mtDNA encodes 13 components of the electron transfer chain, 22 tRNAs and two rRNAs (12S and 16S) which are required for mitochondrial mRNA expression. Mitochondrial DNA also encodes genes involved in other complexes, not including complex II. Mitochondrial complex II succinate dehydrogenase, succinate:ubiquinone oxidoreductase is the only respiratory chain which is entirely coded by nuclear genes. Complex II is involved in the Krebs cycle and in the aerobic electron transfer chain. In the Krebs cycle, Complex II catalyzes the oxidation of succinate to fumarate (Baysal et al., 2001). Every mitochondrion typically has at least

one copy of the mitochondrial genome, and a single mitochondrion can carry as many as ten copies. Cells with higher demands of ATP typically have more mtDNA copies (St John et al., 2005a). Similar to humans, pig mtDNA is 16.6 kb long and encodes 13 subunits of the electron transfer chain, 22 tRNAs and two rRNAs required for mRNA expression (Spikings et al., 2007). The mtDNA rearrangements such as deletions and point mutations may have consequences for spermatogenesis and fertility. Examples include an infertile patient that had a maternally inherited A3243G mutation and two patients with multiple mtDNA deletions correlated with lower sperm motility (Spiropoulos et al., 2002; St John et al., 2005a). Surprisingly, more mtDNA copies seem to be present in spermatozoa of subfertile men (May-Panloup et al., 2003).

Mitochondrial Biogenesis, Structure and Function in Spermatozoa

Sperm mitochondria generate energy for sperm flagellar motility through oxidative phosphorylation (Ruiz-Pesini et al., 1998). Sperm mitochondria are arranged in a helix wrapped around the axoneme-outer dense fiber complex in the sperm tail midpiece (Diez-Sanchez et al., 2003b). Abnormal assembly of the sperm mitochondrial sheath during spermiogenesis has been associated with infertility. The localization and ultrastructure of the mitochondrial sheath is shown in Figure 1. The presence of an abnormally short midpiece has been observed in teratospermic patients (Mundy et al., 1995). Sperm mitochondrial volume is correlated with flagellar beat and sperm length (Ruiz-Pesini et al., 1998) as well as with percentage of motile spermatozoa in a semen sample (Auger et al., 1993; Gopalkrishnan et al., 1991). Sperm cryopreservation may

promote an apoptosis-like state possibly induced by damage to sperm mitochondria (Martin et al., 2007). Normal mitochondrial function is also required for earlier stages of mammalian spermatogenesis prior to formation of the mitochondrial sheath (Nakada et al., 2006).

The mitochondrial genome operates in a semi-autonomous manner as it is co-regulated by nuclear transcription factors. TFAM is a key transcription factor. During spermatogenesis in the mouse, TFAM is expressed up to the late spermatocyte and early spermatid stage. Then an alternative-splicing isoform of TFAM is expressed that lacks a targeting sequence for mitochondria and is targeted to nucleus (Larsson et al., 1996). Human TFAM (hTFAM) mRNA is not differentially spliced in human testis but its expression is downregulated. hTFAM was located in interstitial cells and in basal cells (spermatogonia) of the seminiferous tubules, but this was not visualized in cells at later stages of spermatogenesis (Larsson et al., 1997b). This expression pattern could account for the ten-fold reduction of mtDNA copy number that occurs during late stages of spermatogenesis (Hecht et al., 1984). Northern blot analysis was used to determine which TFAM transcripts are represented in the mouse testis. Testis-specific transcripts approximately of 1 to 1.5 kb in size were found, corresponding to cloned testis cDNA (Larsson et al., 1997b). TFAM protein isoform of 25 kDa is present in both testis and somatic tissues and a larger nuclear isoform of 26 kDa is only present in the testis (Larsson et al., 1997b). Testis-specific human TFAM isoform can be detected in round/elongated spermatids (Larsson et al., 1997b). Altogether, TFAM protein is shown to be down-regulated during human spermatogenesis (Larsson et al., 1997b).

Mitochondrial Inheritance, Function, Biogenesis and Distribution during Oocyte Maturation, Fertilization, Parthenogenesis and Somatic Cell Nuclear Transfer

Mitochondrial function and proper distribution is required for mammalian oocyte maturation. Mitochondrial distribution prior to oocyte maturation in the mouse is radially symmetrical without polarity whereas fully matured oocytes show mitochondrial polarity as relative to the position of metaphase II spindle in the cortex (Calarco, 1995). By Calarco's account, mouse oocyte mitochondria were positioned approximately 30 to 45 degrees on one side of the metaphase II spindle and the polar body I extrusion. Large foci of the mitochondria were found around the center of mature metaphase-II oocytes whereas the central region of the oocyte had fewer mitochondria. In general, most mitochondria were localized in the hemisphere of the metaphase II spindle (Calarco, 1995). In pig *in vitro* matured oocytes, mitochondria translocate primarily to the perinuclear/perichromosomal region during the process of meiotic progression from germinal vesicle breakdown to anaphase I (Schatten et al., 2005). *In vivo* matured porcine oocytes had large mitochondrial foci distributed throughout the cytoplasm. Porcine oocytes matured *in vitro* showed irregular mitochondrial distribution in half of the oocytes examined (Schatten et al., 2005). Rat oocyte mitochondria accumulate in the perinuclear region and travel to the cortex during oocyte maturation (Schatten et al., 2005). In the hamster, mitochondria regroup from a homogeneous dispersed distribution in the oocyte and early pronuclear stage to perinuclear clustering in the advanced pronuclear stage and 2-cell stage. Bovine oocyte and embryo mitochondria accumulated in the cortical and perinuclear areas (Schatten et al., 2005).

Mitochondrial distribution in oocytes is thought to be affected by the oocyte age and culture conditions. It was reported that aged mouse oocytes cannot maintain proper intracellular ATP levels during fertilization (Igarashi et al., 2005). Developmental potential of mouse oocytes can be inferred by mitochondrial distribution wherein high developmental potential is thought to be associated with a homogeneous distribution, and low developmental potential is noted in oocytes with perinuclear mitochondrial clusters (Muggleton-Harris and Brown, 1988). Generally, homogeneous ooplasmic mitochondrial distribution indicates high developmental potential in many species. Such findings suggest that the distribution of mitochondria can be a selection criterion in preparation of mammalian oocytes for micromanipulation procedures (Schatten et al., 2005).

The general rule of strictly maternal inheritance of mitochondria and mtDNA in mammals is thought to be enforced by targeted proteolysis of paternal, sperm contributed mitochondria at fertilization (Ankel-Simons and Cummins, 1996; Sutovsky et al., 1999). One exception is the interspecific hybrids of domestic mouse and Spanish wild mouse (*Mus musculus x Mus spretus*). In the intraspecific strain crosses of *Mus musculus*, paternal mtDNA is only observed in early pronuclear stage and disappears soon after the membrane potential in sperm-derived mitochondria disappears. Interspecific hybrid offspring between *Mus musculus* and *Mus spretus* carry detectable paternal mtDNA (Kaneda et al., 1995). The oocyte cytoplasm may have a specific mechanism for species specificity that recognizes and destroys mitochondria from the sperm as well as the mtDNA. Sperm mitochondria of backcrossed offspring, carrying *M. spretus* mtDNA in mitochondria composed of membrane proteins encoded by *M. musculus* genes, are

recognized by ooplasmic mitochondrial degradation machinery of *M. musculus* ova (Kaneda et al., 1995; Shitara et al., 2000). Therefore, the species-specific mechanism for the degradation of paternal, sperm-contributed mitochondria recognizes the nuclear encoded proteins in the sperm mitochondria rather than the mtDNA itself (Kaneda et al., 1995). Uniparental inheritance is thought to be advantageous in order to prevent continuation of damaged mitochondrial genomes that could be exposed to a mutation-conducive environment during sperm transport in the female reproductive system (Hurst and Hoekstra, 1994).

Mitochondrial distribution, inheritance, and function may also be reflective of developmental potential of mammalian embryos reconstructed by somatic cell nuclear transfer (SCNT). Three possible fates await donor cell and recipient cytoplasm-derived mitochondria in the reconstructed embryos. Homoplasmy will occur where the mitochondria are solely inherited from the recipient oocyte without a donor cell contribution. This pattern is also typical of embryos fertilized by a spermatozoon. In turn, homoplasmy can also occur if all mitochondria of the reconstructed embryo are derived from the donor cell, i.e. when the recipient oocyte mitochondria are eliminated. However, this mode has yet to result in living offspring (Schatten et al., 2005). A Third alternative is heteroplasmy where both the recipient and donor cell mitochondria survive and contribute to embryo development (Schatten et al., 2005).

Cloning of animals has many applications in the agriculture and medicine, including but not limited to propagation of rare, valuable genomes and xenotransplant production. However, the efficiency of cloning remains low with only 0.1% to 5% of cloning attempts resulting in live offspring. One possible reason of the failure of

producing cloned offspring may be due to aberrant mitochondrial distribution resulting in decreased ATP generating capacity and aberrant calcium metabolism. This may cause inability to sustain normal cell functions and to attain normal morphology in embryos (Schatten et al., 2005). Close attention has been paid to mitochondria in the remodeling process and cytoplasm influence on the donor cell nucleus. Recipient cytoplasm-mitochondria may have a principal role in donor cell nuclear remodeling since mitochondria interact directly and indirectly with donor cell nuclei (Schatten et al., 2005). Mitochondria only encompass approximately 1% of the entire genetic material contributed by the recipient ooplasm to the reconstructed embryo. Low developmental potential of SCNT-embryos may result from nuclear-cytoplasmic interactions determined by mitochondria. Nuclear genome-encoded mitochondrial regulatory factors interact with mtDNA and associated mitochondrial-genome derived factors for the normal function and differentiation of mitochondria. Mitochondrial abnormalities due to irregular communication between the nucleus and mitochondria may activate apoptosis and shut down embryo development after SCNT. Overproduction of ATP may create a toxic environment for the SCNT-embryo (Schatten et al., 2005). Disproportional mitochondrial segregation during cell cleavage could result in death of blastomeres and fragmentation of the embryo. Somatic cell nuclear transfer (or SCNT) may thus alter typical mitochondrial distribution patterns (Schatten et al., 2005).

The mtDNA-nuclear DNA interactions could potentially result in altered phenotype of the offspring. Normally, the maternal inheritance occurs in that the oocyte donates all mitochondria to the embryo and the sperm mitochondria are destroyed (Schwartz and Vissing, 2002; Schwartz and Vissing, 2003; Sutovsky, 2004; Sutovsky et

al., 2003a; Sutovsky et al., 1999; Sutovsky et al., 2003b). Cloned embryos display ambiguous mitochondrial inheritance. Heteroplasmy can occur in that offspring from the embryo can have two populations of mtDNA, one from the recipient oocyte and the other from the donor cell mitochondria. Homoplasmy could occur where one population of mtDNA from the donor cell mitochondria or the recipient cell mitochondria prevails. Heteroplasmy can also result from point-mutated or deleted mtDNA molecules that fail to be degraded and trigger mtDNA diseases (Schatten et al., 2005; St John et al., 2005a).

Interspecies cloning/SCNT provides a unique opportunity to study the effects of mitochondrial heteroplasmy. Utilizing bovine oocyte cytoplasm as a recipient for sheep donor cells, the development of embryos from interspecies SCNT can be sustained beyond implantation stage but not to term (Dominko et al., 1999; Scheffler, 2001). The incompatibility of mitochondrial components in the recipient cytoplasm with nuclear encoded mitochondrial factors (such as TFAM) derived from donor cell genome, is a possible reason for lethality of those embryos (Hiendleder, 2007).

Mitochondrial Dysfunction and Infertility

Infertility is a common problem affecting approximately 10 to 15% of couples in the United States (Jose-Miller et al., 2007). Inferior developmental potential of human oocytes from super-stimulated ovarian cycles and aberrant embryo development are thought to be caused by mitochondrial dysfunction. Mouse models of mitochondrial dysfunction demonstrated that oocyte-derived mitochondria regulate embryo growth which can have implications of infertility treatment in women utilizing reproductive

technologies (Thouas et al., 2004). Ooplasm has effects on fertilization of oocytes and early embryo development. Aged women can have a decrease of ATP and mtDNA content, as well as mtDNA damage leading to low reproductive success. Ooplasmic transfers can increase the fertilization ability of aged oocytes and have led to birth of several children (Sills et al., 2004). However, ooplasm donation can result in heteroplasmy (Li and Zhang, 2004) and serious birth defects, and has been banned in the USA (Barritt et al., 2001a; Barritt et al., 2001b).

Male infertility is a common problem among men affecting an estimated 40% of infertile couples. Approximately half of these cases are directly related to sperm motility (Baker, 1994). Oxidative metabolism is a key factor for sperm motility (Ruiz-Pesini et al., 2000). It has been suggested that irregularities in mitochondrial function may result in male infertility (Cummins et al., 1994) possibly due to mitochondrial respiration defects (Nakada et al., 2006). Additionally irregularities in sperm function and structure have been found in patients with mitochondrial myopathies (Folgero et al., 1993). Infertile men have been found to have a higher frequency mtDNA mutations (Spiropoulos et al., 2002) and mtDNA point deletions (Hudson and Chinnery, 2006) in their spermatozoa. Mutation-driven heteroplasmy (not to be confused with biparental mtDNA inheritance) is common in individuals with mtDNA disease since they carry both mutant and wild-type mtDNA. Reduced sperm motility has been correlated with mtDNA mutations (Spiropoulos et al., 2002).

According to the World Health Organization (WHO) guidelines, diagnosis of male infertility is based on sperm concentration, motility, and morphology. However, conventional light-microscopic semen analysis fails to address the function of

mitochondria and mtDNA in male infertility as well as the presence of mtDNA rearrangement that could affect sperm motility and mitochondria (St John et al., 2005a). Experiments have been conducted with mitochondrial inhibitors to assess the importance of oxidative phosphorylation (OXPHOS) in sperm motility (Folgero et al., 1993). It was reported that a patient with poor sperm motility carried a maternally inherited point mutation. However, when the semen of a patient with low sperm motility was supplemented with succinate, which is a metabolite associated with complex II of the electron transfer chain, sperm motility increased (Folgero et al., 1993). An increase in mitochondrial matrix, thicker membranes, and parallelization of cristae and lipid inclusions were found in the patient. These all correspond to mitochondrial disorder symptoms. Irregular mitochondria were also observed in the spermatids. This suggests that mitochondrial dysfunction can result in infertility in men (Folgero et al., 1993). Spermatozoa in a 2 mM glucose environment which mimics the female reproductive tract environment supplemented with OXPHOS inhibitor Rotenone that inhibits complex I, complex IV, and complex V show reduced motility (Ruiz-Pesini et al., 2000). It has been suggested that haplotypes are based on single or short variation in mtDNA typically found in the mtDNA D-Loop, may compromise sperm motility (St John et al., 2005a).

Mitochondrial transcription factor A (TFAM)

The majority of structural mitochondrial proteins are encoded by nuclear genes and synthesized in the cytosol, then imported into the mitochondria (Scheffler, 2001). TFAM is no different in this respect (Chabi et al., 2005). Mitochondrial transcription

factor A, also referred to as TFAM or mtTFA, is a transcriptional and mitochondrial DNA (mtDNA) maintenance factor. This 25 kDa protein is similar to high mobility group proteins (HMG) in that it can bend and unwind mtDNA for the purpose of transcriptional stimulation (Fisher and Clayton, 1988). TFAM protein consists of an amino-terminal HMG domain, a basic linker region, a second HMG domain, and a basic carboxy-terminal tail (Ekstrand et al., 2004). TFAM is highly abundant in the mitochondrial matrix and therefore localized to cytoplasmic regions where mitochondria are present. TFAM is also thought to have architectural properties when acting on mtDNA (Kanki et al., 2004). TFAM function is supported by TFMB1 and TFMB2 which both possess a main function of basal transcription of mammalian mtDNA (Falkenberg et al., 2002). TFAM also plays a critical role in the stabilization and maintenance of mitochondrial chromosomes by phased, sequence- binding to heavy and light strand promoter sites HSP and LSP in the D-Loop of human mtDNA, a control region that regulates transcription and replication (Clayton, 1998; Ekstrand et al., 2004). TFAM overexpression directly increases the mtDNA copy number (Scarpulla, 2006). Therefore TFAM is identified as a major mitochondrial transcription factor controlling mtDNA copy number and mtDNA transcription activity (Appleyard et al., 2006). Cellular TFAM content has been estimated to be at 1 molecule of TFAM per 1000 base pairs of mtDNA in human tissues. Other reports have indicated that TFAM may be present at a ratio of one TFAM molecule per 10 base pairs of mtDNA (Ekstrand et al., 2004). Heterozygous *Tfam* knockout mice (*Tfam*^{+/-}) display a 35 to 40% reduction of mtDNA copy number whereas homozygous knockout embryos of TFAM (*Tfam*^{-/-}) die between E8.5 to E10.5 due to severe respiratory chain deficiency (Ekstrand et al., 2004;

Larsson et al., 1998). TFAM protein depletion is thought to occur between fertilization and the blastocyst stage since the paternally-derived TFAM protein is still present in the ooplasm as the TFAM heterozygous knockout mice have reduced mtDNA copy number but normal oogenesis (Larsson et al., 1998). TFAM protein has been found to be deficient in many mitochondrial myopathies with mitochondrial DNA depletion (Muller-Hocker et al., 1998). Decreased expression of TFAM can result in mtDNA depletion syndromes (Larsson et al., 1994; Poulton et al., 1994). TFAM is an essential regulatory factor in mammalian mitochondrial biogenesis.

Upstream Factors Regulating *Tfam* Gene Expression and TFAM Protein Function

The *Tfam* gene is controlled predominantly by peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) and nuclear respiratory factor-1 (NRF-1) (Figure 2) (Scarpulla, 2006). PGC-1 α is part of the PGC-1 family of coactivators also including PGC-1 β and PRC. This family of transcriptional coactivators is responsible for mitochondrial biogenesis (Wu et al., 1999). PGC-1 α is responsible for coordination and biogenesis of nuclear-encoded mitochondrial transcription factors, such as TFAM. Conditions that increase mitochondrial energy production, such as fasting and exercise training, elevate *PGC-1 α* gene expression (Lin et al., 2005; Puigserver, 2005). Homozygous *PGC-1 α* knockout mice are viable and fertile, unlike TFAM homozygous knockout mice (Scarpulla, 2006). This viability is thought to be attributed to the related *PGC-1 β* gene compensating for the absence of PGC-1 α since PGC-1 β has a similar sequence for the amino acid terminal (Andersson and Scarpulla, 2001). Similar to PGC-

1 α , PGC-1 β mice are viable and fertile and do not demonstrate any irregular phenotype. The PGC-1 β knockout mice do exhibit an altered expression of many nuclear-encoded genes that regulate mitochondrial metabolism in tissues such as heart, skeletal muscle, brain, brown adipose tissue, and liver (Sonoda et al., 2007). While PGC-1 α knockout mice are hyperactive, they have reduced muscle performance, thermogenic defects during cold exposure, cardiac myopias, and defects in metabolism and behavior (Leone et al., 2005; Lin et al., 2004). PGC-1 α stimulates respiratory subunits of mRNAs, COXIV, cytochrome c, and steady state mtDNA levels. PGC-1 α directly interacts with NRF-1 and transactivates NRF-1 targeted genes that are involved in mitochondrial respiration (Kressler et al., 2002; Wu et al., 1999).

NRF-1 is a positive regulator of transcription and acts on the majority of nuclear encoded genes responsible for mitochondrial function; such as TFAM, TFB1M, TFB2M, and POLRMT. (Baar, 2004; Hoppeler and Fluck, 2003). TFAM (Larsson et al., 1998) and NRF-1 (Huo and Scarpulla, 2001) knockout mice die during embryonic development. These knockout embryos are severely depleted of mtDNA. Unlike PGC-1 α , TFAM and NRF-1 are unique. PGC-1 α is part of a small family of related factors whose members may compensate for each other's activity (Scarpulla, 2006). The homozygous *NRF-1* knockout dies between embryonic days 3.5 to 6.5. NRF-1 depletion affects expression of NRF-1 target genes, such as TFAM, that are required for cell growth and development (Larsson et al., 1998; Scarpulla, 2006).

Hypothesis 1: TFAM is Removed from the Sperm Mitochondria During Porcine Spermiogenesis to Reduce the Likelihood of Propagation of Paternal Mitochondria and Paternal mtDNA

In most animal species, paternal mitochondrial inheritance is rare. Organisms such as the blue mussel (Cogswell et al., 2006) and *Chlamydomonas* (Aoyama et al., 2006) inherit paternal mitochondria, but most mammals inherit mitochondria and mtDNA maternally (Birky, 1995; Kuroiwa, 1991), with paternal mitochondrial inheritance being a rare event, as proposed in sheep (Zhao et al., 2004). Early theories attempted to explain maternal mtDNA inheritance by the dilution of paternal mtDNA in the oocyte. Depending on the species, a single spermatozoon contains approximately 100 to 1,000 copies of mtDNA compared to a single oocyte possessing 10^5 to 10^8 copies of mtDNA (Birky, 2001; Diez-Sanchez et al., 2003a; Hecht et al., 1984; Jansen and de Boer, 1998). The cell's requirement for ATP is typically indicated by the number of mitochondria present (Moyes et al., 1998). Oocytes have approximately 100,000 mitochondria compared to the mature sperm possessing 75 to 100 mitochondria (St John et al., 2005a). More recent hypotheses on the mechanism of maternal mitochondrial inheritance focus on targeted degradation of sperm contributed mitochondria inside the zygotic cytoplasm after fertilization. By utilizing Japanese Medaka (*Oryzias latipes*), it was observed that there is marked reduction in the number of mitochondrial nucleoids after fertilization (Nishimura et al., 2006). It has been recently reported that paternal mitochondria in the fertilized egg are specifically eliminated by ubiquitin-proteasome dependent proteolysis (Sutovsky, 2004; Sutovsky et al., 1999). Post-fertilization, sperm mitochondria are incorporated in the oocyte and typically elimination of sperm mitochondria occurs prior to the 8-cell stage by ubiquitin-mediated processes (Sutovsky et al., 1999; Sutovsky et al.,

1996). Similarly, intracytoplasmic injection of a round spermatid into oocytes shows that male germ cell-contributed mitochondria are eliminated (Cummins et al., 1998). In a rare case where paternal mtDNA was reported to be inherited in a human, the patient exhibited mitochondrial myopathy due to a mutation in his mtDNA (Schwartz and Vissing, 2002). It has been shown that during interspecies fertilization of domestic cow oocytes with gaur (*Bos gaurus*) spermatozoa, the ubiquitin-proteasome pathway fails to eliminate paternal mitochondria (Sutovsky et al., 1999).

TFAM has a defined role in spermatogenesis. In the mouse, TFAM is expressed up to the late spermatocyte to early spermatid stage. A testis specific TFAM isoform that lacks the mitochondrial targeting sequence, is targeted to the nucleus (Larsson et al., 1996). In humans, TFAM expression in the testis is different as the alternatively-spliced TFAM species is not present and the constitutive TFAM transcript is down-regulated during spermiogenesis (Larsson et al., 1997b). Higher mtDNA content is found in the sperm of infertile men. Perhaps the deregulation of mtDNA copy number is a marker of spermatogenic dysfunction that may explain some cases of infertility in men (St John et al., 2005a). Reduced levels of human TFAM in testis compared to somatic tissues may be due to transcriptional interference between promoters for testis-specific transcript isoforms with exon I or exon II and somatic transcript isoforms with exon I leading to decreased levels of somatic transcript isoforms and low TFAM content in human germ cell mitochondria. Inconsequently, in humans the testis-specific TFAM transcript with exon I or exon II is not translated into a protein. The levels of TFAM transcript and protein in testis corresponds inversely with somatic cells (Larsson et al., 1997b). The nuclear form of TFAM is not thought to play any important role during spermatogenesis

in mammals in terms of mitochondria, perhaps suggesting an alternative mechanism for TFAM accumulation in the tail piece of spermatozoa.

Hypothesis 2: Maternal TFAM is Essential for Porcine Pre-Implantation Development

The literature reviewed above indicates that TFAM is necessary for preimplantation embryonic development in the mouse. TFAM is an essential factor as TFAM knockout is embryonic lethal with viability not exceeding embryonic day 10.5. TFAM protein depletion is thought to occur between fertilization and the blastocyst stage. The homozygous knockouts die due to a lack of mtDNA and severe respiratory chain deficiency (Ekstrand et al., 2004). *Tfam* heterozygous knockout mice have reduced mtDNA copy number but do have normal oogenesis. Even though the transcript levels of TFAM in heterozygous TFAM knockout mice were only moderately affected, their mtDNA copy number was greatly reduced (Larsson et al., 1998; Maniura-Weber et al., 2004). TFAM reduction caused a decrease of mitochondrial cytochrome oxidase subunit 1 (COX-1) expression down to 30% of normal expression levels and caused embryonic lethality (Ekstrand et al., 2004). COX-1 is one of the three mitochondrial genome-encoded subunits of the cytochrome c oxidase, and is the terminal member of the mitochondrial inner membrane electron transport chain (Tsukihara et al., 1995). TFAM is believed to be the main nuclear-encoded regulatory factor responsible for mammalian mitochondrial biogenesis. Over-expression of TFAM leads to increased mtDNA copy number; however, mitochondrial mass and respiratory chain capacity is not increased (Ekstrand et al., 2004).

Somatic cell nuclear transfer (SCNT) embryos have a reduced developmental potential and the cloned offspring are prone to postnatal health problems. These pathologies, including respiratory distress, heart failures, circulatory abnormalities, immune dysfunctions, kidney problems, and brain diseases may involve mitochondrial dysfunction (Schatten et al., 2005). Large offspring syndrome after SCNT could be caused by mitochondrial defects (Bertolini et al., 2002; Hesselink et al., 2003; Schatten et al., 2005).

CHAPTER III

MITOCHONDRIAL TRANSCRIPTION FACTOR A (TFAM) IN PORCINE GAMETOGENESIS AND PREIMPLANTATION EMBRYO DEVELOPMENT

Introduction

Mitochondrial diseases are among the most prevalent inherited diseases that affect 1 out of 7634 newborns (Debray et al., 2007). Mitochondrial function affects virtually all body systems, including the reproductive system. Major diseases associated with mitochondrial dysfunction include Parkinson's disease, diabetes mellitus, Kearns-Sayre syndrome, Alzheimer's disease; and the aging process has also been associated with mitochondrial dysfunction (Scheffler 2001; Sanaker, Husebye et al. 2007). Mitochondrial positioning may create ATP and calcium gradients that are mandatory for normal embryo development (Schatten et al., 2005). Furthermore, mitochondria have an essential role in apoptosis which has strong implications for cancer research, developmental biology, and senescence and death (Scheffler, 2001).

The mtDNA must be replicated and transcribed in order for mitochondrial multiplication to occur (Scheffler, 2001). Every mitochondrion typically has at least one copy of the mitochondrial genome and a single mitochondrion can carry as many as ten copies. Cells with higher demands of ATP typically have more mtDNA copies (St John et al., 2005a). The mtDNA rearrangements such as deletions and point mutations may have consequences for spermatogenesis and fertility. Examples included an infertile

patient that had a maternally inherited A3243G mutation and two patients with multiple mtDNA deletions that may have resulted in lower sperm motility (Spiropoulos et al., 2002; St John et al., 2005a). Surprisingly, more mtDNA copies seem to be present in spermatozoa of subfertile men compared to fertile donors' spermatozoa (May-Panloup et al., 2003). The mitochondrial genome operates in a semi-autonomous manner as it is co-regulated by nuclear transcription factors. The majority of structural and regulatory mitochondrial proteins are encoded by nuclear genes and synthesized in the cytosol, then imported into the mitochondria (Scheffler, 2001). One of these factors, TFAM plays a primary role in stability and maintenance of mtDNA (Fisher and Clayton, 1988) as well as regulation of mtDNA copy number (Scarpulla, 2006). This 25 kDa protein is similar to high mobility proteins (HMG) in that it can bend and unwind mtDNA to prime it for transcription (Fisher and Clayton, 1988) Kanki et al., 2004 (Kanki et al., 2004). TFAM protein consists of an amino-terminal HMG domain, a basic linker region, a second HMG domain, and a basic carboxy-terminal tail (Ekstrand et al., 2004). TFAM is highly abundant in the mitochondrial matrix and therefore co-localized with mitochondria. TFAM function is supported by transcription factors TFMB1 and TFMB2 which both help to maintain basal transcription of mammalian mtDNA (Falkenberg et al., 2002). TFAM also plays a critical role in the stabilization and maintenance of mtDNA by phased, sequence- binding to heavy and light strand promoter sites (HSP and LSP, respectively) in the D-Loop of human mtDNA, a control region that regulates mtDNA transcription and replication (Clayton, 1998; Ekstrand et al., 2004). TFAM overexpression directly increases the mtDNA copy number (Scarpulla, 2006). Therefore TFAM is identified as a major mitochondrial transcription factor controlling mtDNA

copy number and mtDNA transcription activity (Appleyard et al., 2006). Cellular TFAM content has been estimated by some to be at 1 molecule of TFAM per 1000 base pairs of mtDNA in human tissues. Other reports have indicated that TFAM may be present at a ratio of one TFAM molecule per 10 base pairs of mtDNA (Ekstrand et al., 2004). Heterozygous *Tfam* knockout mice (*Tfam*^{+/-}) display a 35-40% reduction of mtDNA copy number whereas homozygous knockout embryos of TFAM (*Tfam*^{-/-}) die between E8.5 to E10.5 due to severe respiratory chain deficiency (Ekstrand et al., 2004; Larsson et al., 1998). TFAM protein depletion is thought to occur between fertilization and the blastocyst stage since the maternally-derived TFAM protein is still present in the ooplasm. Consequently, the TFAM heterozygous knockout mice have reduced mtDNA copy number but normal oogenesis (Larsson et al., 1998). TFAM protein has been found to be deficient in many mitochondrial myopathies associated with mitochondrial DNA depletion (Muller-Hocker et al., 1998). Decreased expression of TFAM can result in mtDNA depletion syndromes (Larsson et al., 1994; Poulton et al., 1994). TFAM is an essential regulatory factor in mammalian mitochondrial biogenesis.

Mitochondrial function and proper distribution is required for mammalian oocyte maturation. Mitochondrial distribution prior to oocyte maturation in the mouse is radially symmetrical without polarity whereas fully matured oocytes show mitochondrial polarity as relative to the position of the metaphase II spindle in the cortex (Calarco, 1995). In pig *in vitro* matured oocytes, mitochondria translocate primarily to the perinuclear or perichromosomal region during the process of meiotic progression from germinal vesicle breakdown to anaphase I (Schatten et al., 2005). *In vivo* matured porcine oocytes have large mitochondrial foci distributed throughout the cytoplasm. Porcine oocytes matured

in vitro show irregular mitochondrial distribution in half of the oocytes examined (Schatten et al., 2005). Mitochondrial distribution in oocytes is thought to be affected by the oocyte age and culture conditions. Igarashi et al. reported that aged mouse oocytes cannot maintain proper intracellular ATP levels during fertilization (Igarashi et al., 2005). Generally, homogeneous ooplasmic mitochondrial distribution indicates high developmental potential in many species. Such findings indicate that the distribution of mitochondria can be used as a selection criterion in preparation of mammalian oocytes for micromanipulation procedures (Schatten et al., 2005).

Mitochondrial distribution, inheritance and function may also be reflective of developmental potential of mammalian embryos reconstructed by somatic cell nuclear transfer (SCNT). Three possible fates await donor cell and recipient cytoplasm-derived mitochondria in the reconstructed embryos. Homoplasmy will occur where the mitochondria are solely inherited from the recipient oocyte without a donor cell contribution. This pattern is also typical of embryos fertilized by a spermatozoon. In turn, homoplasmy can also occur if all mitochondria of the reconstructed embryo are derived from the donor cell, i.e. when the recipient oocyte mitochondria are eliminated. However, this mode has yet to result in living offspring (Schatten et al., 2005). A third alternative is heteroplasmy where both the recipient and donor cell mitochondria survive and contribute to embryo development (Schatten et al., 2005).

Somatic cell nuclear transfer (SCNT) has many applications in the agriculture and medicine, including but not limited to propagation of rare, valuable genomes and xenotransplant production. However, the efficiency of SCNT remains low with only 0.1% to 1% of cloning attempts resulting in live offspring. Cloned embryos have a reduced

developmental potential and the cloned offspring are prone to postnatal health problems. These pathologies, including respiratory distress, heart failures, circulatory abnormalities, immune dysfunctions, kidney problems and brain diseases may involve mitochondrial dysfunction (Schatten et al., 2005). Large offspring syndrome after SCNT could be caused mitochondrial defects (Hesselink et al., 2003; Schatten et al., 2005). One possible reason of the failure of producing cloned offspring may be due to aberrant mitochondrial distribution resulting in decreased ATP generating capacity and aberrant calcium metabolism. This may cause inability to sustain normal cell functions and to attain normal morphology in embryos (Schatten et al., 2005). Close attention has been paid to mitochondria with regard to the remodeling process and cytoplasm influence on the donor cell nucleus. Mitochondria only encompass approximately 1% of the entire genetic material contributed by the recipient ooplast to the reconstructed embryo. Low developmental potential of SCNT-embryos may be caused by nuclear-cytoplasmic interactions determined by mitochondria. Nuclear genome-encoded mitochondrial regulatory factors interact with mtDNA and associated mitochondrial-genome derived factors for normal function and differentiation of mitochondria. Mitochondrial abnormalities due to irregular communication between nucleus and mitochondria may activate apoptosis and shut down embryo development after SCNT. Overproduction of ATP may create a toxic environment for the SCNT-embryo (Schatten et al., 2005). Disproportional mitochondrial segregation during cell cleavage could result in death of blastomeres and fragmentation of the embryo. Somatic cell nuclear transfer may thus alter typical mitochondrial distribution patterns (Schatten et al., 2005).

Infertility is a common problem affecting approximately 10-15% of couples in the United States (Jose-Miller et al., 2007). Inferior developmental potential of human oocytes from super-stimulated ovarian cycles and aberrant embryo development are thought to be caused by mitochondrial dysfunction (Thouas et al., 2004). Mother's age has a profound effect on fertilization and early embryo development following assisted fertilization. Aged oocytes have a reduced ATP and mtDNA content, as well as mtDNA damage leading to low reproductive success. Ooplasmic transfers can increase the fertilization ability of aged oocytes and has led to the birth of several children (Sills et al., 2004). However, ooplasm donation can result in heteroplasmy (Li and Zhang, 2004) and serious birth defects, and has been banned in USA (Barritt et al., 2001a; Barritt et al., 2001b).

Male infertility is a common problem among men affecting an estimated 40% of infertile couples. Approximately half of these cases are directly related to sperm motility (Baker, 1994). Oxidative metabolism is a key factor for sperm motility (Ruiz-Pesini et al., 2000). Sperm mitochondria generate energy for sperm flagellar motility through oxidative phosphorylation (Ruiz-Pesini et al., 1998). Abnormal assembly of the sperm mitochondrial sheath during spermiogenesis has been associated with infertility. The presence of an abnormally short midpiece has been observed in teratospermic patients (Mundy et al., 1995). Normal mitochondrial function is also required for earlier stages of mammalian spermatogenesis prior to formation of the mitochondrial sheath (Nakada et al., 2006). Irregularities in mitochondrial function may result in male infertility (Cummins et al., 1994) possibly due to mitochondrial respiration defects (Nakada et al., 2006). Additionally irregularities in sperm function and structure have been found in

patients with mitochondrial myopathies (Folgero et al., 1993). Infertile men have been found to have a higher frequency of mtDNA mutations (Spiropoulos et al., 2002) and mtDNA point deletions (Hudson and Chinnery, 2006) in their spermatozoa. Mutation-driven heteroplasmy (not to be confused with biparental mtDNA inheritance) is common in individuals with mtDNA disease since they carry both mutant and wild-type mtDNA. Reduced sperm motility has been correlated with mtDNA mutations (Spiropoulos et al., 2002). TFAM likely has a role in spermatogenesis. In the mouse, TFAM is expressed up to the late spermatocyte to early spermatid stage. A testis specific TFAM isoform that lacks the mitochondrial targeting sequence is targeted to the nucleus (Larsson et al., 1996). In humans, TFAM expression in the testis is different as the alternatively-spliced TFAM species is not present and the constitutive TFAM transcript is down-regulated during spermiogenesis (Larsson et al., 1997b). Higher mtDNA content is found in the spermatozoa of infertile men. Perhaps the deregulation of mtDNA copy number is a marker of spermatogenic dysfunction that may explain some cases of infertility in men (St John et al., 2005a). Reduced levels of human TFAM in testis compared to somatic tissues may be due to transcriptional interference between promoters for testis-specific transcript isoforms and somatic transcript isoforms leading to decreased levels of somatic transcript isoforms and low TFAM content in human germ cell mitochondria. Consequently, the human testis-specific TFAM transcript is not translated into a protein. The level of TFAM transcript and protein in testis corresponds inversely with somatic cells (Larsson et al., 1997b). This TFAM expression pattern could account for the ten-fold reduction of mtDNA copy number that occurs during late stages of spermatogenesis (Hecht et al., 1984). The nuclear form of TFAM is thought to not play any important

role during spermatogenesis in mammals in terms of mitochondria perhaps suggesting an alternative mechanism for TFAM accumulation in the tail piece of sperm.

In most animal species, paternal mitochondrial inheritance is rare. Organisms such as the blue mussel (Cogswell et al., 2006) and *Chlamydomonas* (Aoyama et al., 2006) inherit paternal mitochondria but most mammals inherit mitochondria and mtDNA maternally (Birky, 1995; Kuroiwa, 1991), with paternal mitochondrial inheritance being a rare event, as proposed in sheep (Zhao et al., 2004). Early theories attempted to explain maternal mtDNA inheritance by the dilution of paternal mtDNA in the oocyte. Depending on the species, a single spermatozoon contains approximately 100 to 1000 copies of mtDNA compared to a single oocyte possessing 10^5 to 10^8 copies of mtDNA (Birky, 2001; Diez-Sanchez et al., 2003a; Hecht et al., 1984; Jansen and de Boer, 1998). The cell's requirement for ATP is typically indicated by the number of mitochondria present (Moyes et al., 1998). Oocytes have approximately 100,000 mitochondria compared to the mature sperm possessing 75 to 100 mitochondria (St John et al., 2005a). More recent hypotheses on the mechanism of maternal mitochondrial inheritance focus on targeted degradation of sperm contributed mitochondria inside the zygotic cytoplasm after fertilization. By utilizing Japanese Medaka (*Oryzias latipes*), Nishimura et al. observed a marked reduction in the number of mitochondrial nucleoids after fertilization (Nishimura et al., 2006). It has been recently reported that paternal mitochondria in the fertilized egg are specifically eliminated by ubiquitin-proteasome dependent proteolysis (Sutovsky, 2004; Sutovsky et al., 1999) Post-fertilization, sperm mitochondria are incorporated in the oocyte and typically elimination of sperm mitochondria occurs prior to 8-cell stage by a ubiquitination-dependent proteasomal proteolysis (Sutovsky et al.,

1999; Sutovsky et al., 1996). Similarly, male germ cell-contributed mitochondria are eliminated after intracytoplasmic injection of a round spermatid into an oocyte (Cummins et al., 1998). In a rare documented case where paternal mtDNA was reported to be inherited in a human, the patient exhibited mitochondrial myopathy due to a mutation in his mtDNA (Schwartz and Vissing, 2002). It has been shown that during interspecies fertilization of domestic cow oocytes with gaur (*Bos gauras*) spermatozoa, the ubiquitin-proteasome pathway fails to eliminate paternal mitochondria (Sutovsky et al., 1999).

TFAM is necessary for preimplantation embryo development in the mouse. TFAM is an essential factor as a TFAM knockout is embryonic lethal with viability not exceeding embryonic day 10.5. TFAM protein depletion is thought to occur between fertilization and the blastocyst stage. The homozygous knockouts die due to lack of mtDNA and severe respiratory chain deficiency (Ekstrand et al., 2004). *Tfam* heterozygous knockout mice have reduced mtDNA copy number but do have normal oogenesis. Even though the transcript levels of TFAM in heterozygous TFAM knockout mice were only moderately affected, their mtDNA copy number was greatly reduced (Larsson et al., 1998; Maniura-Weber et al., 2004). TFAM reduction caused a decrease of mitochondrial cytochrome oxidase subunit 1 (COX-1) expression down to 30% of normal expression levels and caused embryonic lethality (Ekstrand et al., 2004). COX-1 is one of the three mitochondrial genome-encoded subunits of the cytochrome c oxidase, and is the terminal member of the mitochondrial inner membrane electron transport chain (Tsukihara et al., 1995). TFAM is believed to be the main nuclear-encoded regulatory factor responsible for mammalian mitochondrial biogenesis. Over-expression of TFAM

leads to increased mtDNA copy number, however, mitochondrial mass and respiratory chain capacity is not increased (Ekstrand et al., 2004).

The present study seeks to validate two hypotheses: 1) TFAM is removed from the sperm mitochondria during porcine spermiogenesis to reduce the likelihood of propagation of paternal mitochondria and paternal mtDNA and 2) maternal TFAM is essential for porcine pre-implantation development.

Materials and Methods

Antibodies

The rabbit anti-TFAM polyclonal antibody (IgG) (ProSci Inc.; Poway, CA) was purchased and used for western blotting at a 1:5000 dilution. This antibody was diluted 1:100 for immunocytochemistry. Rabbit poly-clonal β -actin antibody loading control (IgG) was used in western blotting at a concentration of 1:5000 and was purchased from Abcam (Cambridge, UK). Secondary antibodies conjugated to Fluorescein Isothiocyanate (FITC), Rhodamine (TRITC), and Horseradish Peroxidase (HRP) were bought from commercial sources (Zymed Lab Inc., San Francisco, CA). FITC- and TRITC- antibodies were used in concentrations at 1:80 for immunocytochemistry. HRP-conjugated antibodies were used at a concentration of 1:10000 for western blotting.

Boar Ejaculate and Porcine Testicular and Ovarian Tissue Collection

All procedures and experimental protocols were reviewed and approved by the Animal Care and Use Committee (ACUC) of University of Missouri - Columbia.

Animals were housed in facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Boar ejaculates were collected by a gloved hand technique from Large White fertile boars at the South Farm, University of Missouri – Columbia. All boars were between 9 months to 2 years of age when ejaculate was collected. Samples were collected on an as needed basis. Ovaries were obtained from prepubertal gilts at a local slaughterhouse.

Isolation of Boar Sperm Heads and Tails

Ejaculated boar spermatozoa were obtained and washed using 0.9 M phosphate buffered saline (PBS; pH 7.4) and centrifuged at 8,000 RPM in a Centrifuge Benchtop Centrifuge (Model 225; Fisher Scientific; Pittsburgh, PA) for 5 min for a total of three times. Sperm pellets were collected and mixed with cold PBS containing 10 μ L per 1 mL sperm sample of Halt Protease Inhibitor Cocktail (Pierce; Rockford, IL) and 10 mM PMSF. Sperm samples were sonicated with a Branson Digital Sonifier (Branson Ultrasonic Corp, Danbury, CT) at 50% amplitude for one minute. The sperm sample was centrifuged to collect the sperm pellet and was mixed with 85% sucrose in pure PBS. The Sucrose-sperm sample mixture was centrifuged in a Beckman ultracentrifuge for 1 h in 4°C at 100,000 x G by using a Type 75 Ti rotor head (Beckman Coulter; Fullerton, CA). Sperm heads and sperm tails were collected from the centrifuge tube after centrifugation based on differential migration on the sucrose column. The sperm heads accumulated at the bottom of the centrifuge tube and the sperm tails were located on the side by the top of the sucrose column. The sperm head fraction was further purified by repeating this procedure three times followed by an additional centrifugation using the

SW-55 Beckman rotor head for 1 h in 4°C at 100,000 x G (Beckman Coulter; Fullerton, CA). Sperm head and sperm tail fractions were examined under a light microscope to check the purity of the head/tail preparations, and further sonicated and centrifuged until the purity of the sperm-head fraction was more than 99% as assessed by counting 1,000 sperm head/tail portions per sample and comparing the number of separated heads (>99%) with number of separated tails and intact spermatozoa (<1%).

Protein Extraction and Determination of Protein Concentration by Bradford Assay

A two-fold concentrated extraction buffer was prepared containing 100 mM Tris (pH 6.8), 150 mM NaCl, 40 mM imidazole, 2 mM EDTA, and 10 mM Benzamidine HCl. A boar sperm sample was obtained then washed with PBS. The sperm pellets were mixed with the extraction buffer at a 1:1 ratio in cryovials. Halt Protease Inhibitor Cocktail (Pierce; Rockford, IL) was added at 10 µL per 1 mL sperm sample. A 100 mM stock solution of PMSF (Sigma-Aldrich; St. Louis, MO,) was added to the sample at a ratio of 1:100. The suspensions were frozen by plunging into liquid nitrogen for 5 min followed by thawing by dipping in warm water and shaking vigorously for 2 to 3 min. This freeze-thawing was repeated three times. Total protein content was determined by using a Bradford Protein Assay kit (Bio-Rad Laboratories; Hercules, CA) and 2 to 10 µg/ml BSA standards.

SDS-PAGE, Western Blotting and Densitometry

A standard laboratory western blotting protocol was applied (Manandhar et al., 2006). For western blotting, either spermatozoa or oocytes were pelleted, washed with

PBS, and extracted by boiling for 5 min with loading buffer (2% sodium dodecyl sulfate, 20% glycerol, 0.002% bromophenol blue, and 5% β -mercaptoethanol). Sperm extracts were loaded at equal protein concentrations. Unfertilized GV-stage and MII-oocytes were vortexed with 0.5% hyaluronidase to remove the cumulus cells completely. For each lane, 100 oocytes or pre-blastocyst stage embryos were dissolved in 10 μ l loading buffer, boiled for 5 min, and loaded in one lane per sample.

Electrophoresis was performed on 4 to 20% gradient polyacrylamide gel electrophoresis (PAGE) gels and the resolved proteins were transferred to polyvinylidene fluoride (PVDF) membrane using a wet transfer system (Towbin et al., 1979). Washing and incubation were done with TBS containing 0.25% Tween-20 (TBS-TWN) and 1% non-fat milk. The membranes were blocked with 10% non-fat milk in TBS-TWN. They were incubated either with β -actin antibody for one membrane and with TFAM antibody for another membrane overnight at 4°C. After washing, the membranes were incubated with HRP-conjugated goat anti-mouse IgG (10,000 x dilution), processed for chemiluminescence using a commercial kit (SuperSignal, Pierce; Rockford, IL), and visualized by exposing to X-ray film for 1 minute.

Densitometry was performed as previously described (Lovercamp et al., 2007). Image analysis was performed by using Kodak 1-D Image Analysis software (Kodak Scientific Imaging Systems, New Haven, CT).

Affinity Purification of Ubiquitinated Proteins from Boar Sperm Extracts

A total of 50 μ L of agarose conjugated p62-derived ubiquitin-pathway associated (UBA) domain (Biomol International, L.P.; Plymouth Meeting, PA) was mixed with 200 μ L 0.9 M Tris buffered saline 7.4 pH (TBS; 0.05 M Tris base, 0.2 M NaCl dissolved in 1,000 mL ultra pure water) and centrifuged in a Sorvall Biofuge Fresco (Kendro Laboratory Products; Asheville, NC) centrifuge at 3,000 RPM for 1 min at 4°C. The supernatant was removed and an additional 400 μ L of TBS was added to the pellet without mixing. The sperm extract was centrifuged at 13,000 RPM for 10 min, and 100 μ L of sperm supernatant was mixed with p62-agarose and coincubated at 4°C for 24 h on a rocker set to low amplitude and slow speed. After incubation, the sample was centrifuged at 3,000 RPM at 4°C for one minute. The supernatant was removed and the pellet was washed three times with TBS. To elute ubiquitinated proteins, 20 μ L of 2x loading buffer (2% sodium dodecyl sulfate, 20% glycerol, 0.002% bromophenol blue, and 5% β -mercaptoethanol) was added to the p62-agarose with sperm proteins. The sample was boiled for 5 min. The sample was filtered in an Eppendorf filtration tube with size 3 filter paper (Whatman; Brentford, Middlesex, UK) by centrifugation at 13,000 RPM for 10 min at 21°C. The liquid extract was used for western blotting.

Oocyte Maturation

Oocyte and embryo culture procedures were done by following our standard lab protocol (Manandhar et al., 2006). Cumulus–oocyte complexes (COCs) were aspirated from antral follicles (3 to 6 mm size) and washed in HEPES-buffered Tyrode lactate

medium containing 0.1% (w/v) polyvinyl alcohol (TL-HEPES-PVA) for a total of three times. For *in vitro* maturation, the oocytes were transferred to a 500 µl drop of maturation medium (TCM 199; Invitrogen; Carlsbad, CA) supplemented with 0.1% PVA, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 µg/ml luteinizing hormone (LH) (Sigma-Aldrich; St. Louis, MO), 0.5 µg/ml follicle-stimulating hormone (FSH), 10 ng/ml epidermal growth factor, 10% porcine follicular fluid, 75 µg/ml penicillin G, and 50 µg/ml streptomycin. Mineral oil was applied to cover the top of the medium drops in four-well dishes (Nunc; Roskilde, Denmark) and they were incubated at 38.5°C, 5% CO₂ in air. After 22 h of culture, the oocytes were transferred to a maturation medium without LH and FSH, and cultured for an additional 22 h.

In Vitro Fertilization and Embryo Culture

For *in vitro* fertilization, the cumulus cells from the matured COCs were removed by vortexing with 0.1% hyaluronidase in TL-HEPES-PVA medium and washed in a Tris-buffered medium (mTBM) (Abeydeera et al., 1998) containing 0.2% (w/v) BSA. Approximately 30 oocytes were transferred into 50 µl drops of mTBM, covered with mineral oil, then incubated for 30 min until spermatozoa were added. A sperm pellet was thawed in PBS containing 0.1% PVA (PBS-PVA; Sigma-Aldrich; St. Louis, MO) and centrifuged through a two-layer Percoll gradient (60/40%) at 2,500 RPM for 10 min in a IEC Centra CL2 benchtop centrifuge (Thermo Scientific; Waltham, MA). The spermatozoa were resuspended and washed two times in PBS-PVA, and then resuspended in mTBM. The sperm suspension was added to the 50 µl drops of medium containing oocytes for a final sperm concentration of 10⁶/ml. Oocytes were coincubated

with spermatozoa for 6 h, then transferred to 500 μ l drops of NCSU-23 medium (Petters and Wells, 1993) containing 0.4% BSA for additional culture.

Activation of Oocytes for Western Blotting

Parthenogenetic oocyte activation was performed by following an established protocol (Yi and Park, 2005). After *in vitro* maturation, cumulus cells were removed by 0.1% hyaluronidase in mTLP-PVA then washed in HEPES buffered (25 mM) NCSU-23 medium three times. Oocytes with the first polar body were selected and activated in TCM 199 medium with 8% ethanol for 10 min. After that, oocytes were incubated with 4 mM 6-dimethylaminopurine (DMAP) in TCM 199 medium for 6 h at 38.5°C, 5% CO₂ in air. After activation, oocytes were washed in NCSU-23 medium for continued culture.

Embryos Produced by Somatic Cell Nuclear Transfer

Matured oocytes were purchased (BOMED, Inc.; Columbia, Missouri). Cumulus-free (denuded) oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm in enucleation medium with a glass pipette 30 μ m in diameter. The donor cells were injected into the perivitelline space of the oocyte through the same hole in the zona pellucida with the pipette previously used. Injected oocytes were placed between 0.2 mm-diameter platinum electrodes 1 mm apart in a fusion medium. Fusion was induced with 2 DC pulses (1 sec interval) of 1.2 kV/cm for 30 μ sec on a BTX Electro-Cell Manipulator 200 (BTX, San Diego, CA). The medium used for enucleation was tissue culture medium (TCM) 199 supplemented with HEPES, 0.3% BSA, and 7.5 μ g/mL cytochalasin

B (CB), and the medium for injection was the same medium without CB (Kolber-Simonds et al., 2004; Lai et al., 2002).

Oocyte Activation Treatments for Somatic Cell Nuclear Transfer

The following three activation strategies were implemented.

Group NT-1: Electrical activation. Oocyte activation was achieved during fusion by an electrical pulse in fusion/activation PZM3 culture medium (0.3 M mannitol, 1.0 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.5 mM HEPES).

Group NT-2: Transient treatment with a reversible proteasomal inhibitor. After the electrical pulse-induced activation the oocytes were washed with PZM-3 with 0.3% BSA one time, then directly transferred into PZM-3 with 0.3% BSA and 10 μM MG-132. After incubation for 2 h, the fused oocytes were selected and washed in PZM-3 with 0.3% BSA for a total of three times and then cultured in the same medium for up to 144 hours.

Group NT-3: Chemical activation. Reconstructed oocytes were fused in activation medium with a low calcium concentration (0.1 mM). The fused oocytes were treated with 200 μM thimerosal for 10 min. After the oocytes were washed in embryo manipulation medium one time, they were treated with 8 mM dithiothreitol (DTT) for a total of 30 min and cultured in PZM3. (Machaty et al., 1997).

Fixation of Oocytes

Oocytes were suspended in 0.1% hyaluronidase in TL-HEPES-PVA for 5 min for cumulus cell removal. Zonae pellucidae were removed by protease in TL-HEPES-PVA. Oocytes were fixed in 2% formaldehyde in PBS at room temperature (RT) for 40 min in nine-well glass plates (Pyrex brand; Fisher Scientific, Pittsburgh, PA), washed with PBS, and used immediately for immunocytochemistry or stored overnight in PBS at 4°C.

Fixation of Boar Spermatozoa

A standard laboratory protocol for fixation of boar spermatozoa was used (Sutovsky, 2004). Microscopy cover slips (18 x 18 mm) were cleaned in 100% ethanol and coated with 1% w/v poly-L-lysine in ultra pure water (Sigma-Aldrich; St. Louis, MO). Frozen boar sperm pellets or washed ejaculated boar spermatozoa were resuspended in 37°C warm TL-HEPES-PVA. The sample was centrifuged for 5 min at 350 x g. Cover slips were overlaid with 400 µL of warm KMT medium (100 mM KCl, 2 mM MgCl₂·6H₂O, 10 mM Tris-HCl, 5mM EGTA) and 2 µL of sperm sample were added to each cover slip. Cover slips were incubated for 5 min then transferred to 2% formaldehyde in PBS for 40 min fixation. Cover slips with fixed spermatozoa were washed with PBS and processed for immunocytochemistry.

Immunocytochemistry

Samples of oocytes or spermatozoa were submerged in PBS with 0.1% Triton-X-100 (Sigma-Aldrich; St. Louis, MO) for 40 min for permeabilization. Samples were then blocked using 5% normal goat serum (NGS; Sigma-Aldrich; St Louis, MO) for 25 min.

Rabbit anti-TFAM polyclonal antibody (IgG) (ProSci Inc.; Poway, CA) was diluted 1:200 in PBS containing 1% NGS and 0.1% Triton-X, and incubated with samples at 4°C overnight. After washing, samples were incubated for 35 min. with a secondary antibody solution containing 5 µg/ml DAPI and 1:80 dilution of tetramethyl-rhodamine-isothiocyanate-conjugated goat anti-rabbit IgG (GAR-TRITC; Invitrogen; Carlsbad, CA), protected from light. Cover slips or oocytes were then mounted on conventional microscopy slides in VECTASHIELD[®] mounting medium (Vector Laboratories; Burlingame, CA). Slides were examined and photographed by using the Nikon Eclipse E800 microscope with a CoolSNAP *hq* monochrome camera and acquired using MetaMorph v. 4.6.7 software. Images were edited using Adobe[®] Photoshop 5.5 (Adobe Systems, Inc., San Jose, CA).

Densitometry Data Analysis

Optical intensity was computed by $I_{\text{Corrected}} = I_{\text{Lane}} - I_{\text{background}}$ where the intensity was the sum intensity from a given measurement. For comparative state observations the equation $C_{\text{LaneA}} = mC_{\text{LaneB}}$ where Lane B has m times more/less sum intensity than Lane A.

Results

Segregation of TFAM from the Midpiece to the Principal Piece during Spermatid Elongation

Given the importance of sperm mitochondria for sperm function, we examined the localization of TFAM in boar during spermatogenesis, spermiogenesis, and after sperm maturation and ejaculation. Localization patterns were interpreted with regard to known requirements of sperm mitochondrion degradation for the clonal, maternal derivation of embryonic mitochondrial genome in mammals. On the boar testicular tissue sections the TFAM-protein was primarily associated with the cytoplasmic lobes of steps 8-12 elongating spermatids (Figure 4A). Contrary to the expectation, no distinct labeling was observed within the developing mitochondrial sheath of late step elongating spermatids (data not shown).

In the intact ejaculated boar spermatozoa, TFAM was localized in the sperm tail principal piece, but not in the midpiece/mitochondrial sheath (Figure 5A). TFAM labeling was not observed in control samples processed with normal rabbit serum and appropriate secondary antibody (Figure 5B). Western blotting of isolated sperm head and tail extracts obtained by sperm sonication detected the anticipated 25 kDa TFAM band in the sperm tail fraction and whole sperm extract, but not in the isolated sperm head fraction (Figure 6A). Equal protein loading was confirmed by western blotting with the anti- β -actin antibody, showing an distribution of actin bands in all lanes (Figure 6B). In some repeats, TFAM bands were observed in both sperm head and sperm tail extracts, possibly due to TFAM protein release from the sperm tail, binding to the sperm head surface and partial degradation after prolonged sonication (Figure 7A). In addition to

the expected 25 kDa band, sonication produced a partially degraded protein species migrating at approximately 13 kDa (Figure 7A), at the equivalent protein load (Figure 7B). Consequently, immunofluorescence labeling was observed consistently in the principal pieces of the isolated sperm tails (Figure 8C). There was sperm head labeling only in some trials that was observed to be localized to the surface of the postacrosomal sheath (Figure 8A). TFAM signals were not detected in control samples incubated with normal rabbit serum and fluorescently labeled anti-rabbit IgG (Figure 8B, 8D). In summary, consistent TFAM localization is observed in the cytoplasmic lobes of elongating spermatids and in the principal pieces of fully differentiated spermatozoa.

Boar Sperm TFAM Protein is Posttranslationally-Modified by Ubiquitination

In addition to segregation of TFAM from the sperm mitochondrial sheath into the sperm tail principal piece, posttranslational modification of spermatid/sperm TFAM by ubiquitination could contribute to timely degradation of the sperm mitochondria and flagellum after fertilization. Ubiquitination and subsequent proteasomal degradation have been implicated in the targeted degradation of paternal, sperm contributed mitochondria after fertilization in mammals (Sutovsky et al., 2003; Sutovsky et al., 1999). Furthermore, ubiquitination and proteasomal degradation of spermatid proteins are necessary for successful spermatid elongation and biogenesis of sperm accessory structures ((Escalier, 2003; Escalier et al., 2003). To examine TFAM protein-ubiquitination, we isolated ubiquitinated proteins from boar sperm extracts by affinity purification with synthetic UBA domain of ubiquitin binding-protein p62, immobilized on agarose beads. The eluted, ubiquitinated sperm proteins were subsequently probed

with anti-TFAM antibody, revealing 2-3 (dependent on protein load) major bands migrating above the expected 25 kDa TFAM band on SDS-PAGE (Figure 9). The p62-derived UBA domain binds weakly but specifically to multi-ubiquitin chains covalently linked to ubiquitinated proteins, allowing highly specific purification and easy elution of ubiquitinated proteins from any cell extract. While the nascent non-ubiquitinated TFAM band was hardly distinguishable in the p62 –purified preparation, a major band was observed around 55 to 58 kDa, suggesting tetraubiquitination (4×8.5 kDa) of the 24 to 25 kDa sperm TFAM protein. Control sperm extracts coincubated with unconjugated agarose displayed predominantly the non-ubiquitinated, nascent 25 kDa TFAM band, while control elution of p62-agarose not exposed to sperm extract did not yield anti-TFAM immunoreactive bands (Figure 9). Based on these results, TFAM is believed to be tetraubiquitinated in boar spermatozoa.

TFAM Protein Accumulates in the Ooplasm during Oocyte Meiotic Maturation

The number of oocyte mtDNA copies is believed to increase between the GV-stage and MII-stage of oocyte maturation (St John et al., 2005a). To determine if the replication of mtDNA during oocyte maturation is reflected by changes in the amount of ooplasmic TFAM protein, porcine oocytes and embryos were examined by using immunofluorescence labeling of ovarian tissue sections and whole-mounted oocytes, as well as semi-quantitative western blotting technique. On the ovarian tissue sections, predominant TFAM labeling was localized to the ooplasm in the oocyte (Figure 10A). Increased TFAM accumulation was measurable by immunofluorescence labeling of whole-mounted metaphase II (MII) oocytes compared side-by-side on the same slide with

germinal vesicle (GV) stage oocytes (Figure 10B). The pronuclear stage zygotes displayed a diffuse cytoplasmic pattern of TFAM localization (Figure 10C). At day 7 of embryo development, the blastocysts displayed a somatic cell-like, punctate cytoplasmic localization of TFAM protein, corresponding to individual mitochondria in the blastomere cytoplasm (Figure 10D, 10E).

Western blotting of TFAM in GV-stage and MII-stage oocytes showed an increased band density of the predicted 25 kDa TFAM-band in the MII oocytes. The combined TFAM-band density of the MII-stage oocytes was 4.4x greater than that of the GV-stage oocytes (Figure 11A). The 25 kDa band density was greatly diminished in the pronuclear zygotes at 22 h after IVF (Figure 11B). In contrast, parthenogenetic activation of porcine oocytes with 8% ethanol resulted in a 44.6x increase of TFAM band density compared to *in vitro* fertilized embryos cultured for 22 h. The parthenogenetically activated oocytes also had a 2.8x greater band density than that of the MII-stage oocytes (Figure 11B). At the mRNA level, the relative amount of TFAM transcript increased between the GV-stage oocyte (last transcriptionally active stage prior to oocyte maturation) and the 4-cell stage (onset of zygotic genome transcription in pig) (Figure 12). Further increase in TFAM transcripts was observed between 4-cell and blastocyst stage *in vivo*, but not *in vitro*. *In vitro* produced blastocysts actually showed a reduction in TFAM mRNA content as compared to the 4-cell stage. In summary, TFAM protein accumulates in the cytoplasm of porcine oocytes during *in vitro* maturation and an increase in TFAM transcript number is observed after the activation of embryonic genome transcription. Degradation of maternally-stored TFAM protein seems to occur

shortly after fertilization, but parthenogenetically activated oocytes display a much greater accumulation of TFAM protein than *in vitro* fertilized oocytes.

Effects of Different Oocyte Activation Procedures on the Accumulation of TFAM Protein in Porcine Zygotes Generated by Somatic Cell Nuclear Transfer (SCNT)

Recent work on cloned mice revealed altered expression of the *Tfam* gene in cloned embryos, compared to embryos generated by natural fertilization (Vassena et al., 2007). Consequently, we examined the accumulation of TFAM protein by western blotting in cloned porcine zygotes and blastocysts that were produced by three different modifications of standard SCNT procedure. The NT-1 zygotes showed a dominant TFAM-band at the 25 kDa level while a degradation product of approximately 16 kDa was most dominant in NT-3 zygotes. The highest density of the 25 kDa TFAM band was observed in the NT-2 zygotes. In the NT-2 group, the prominent band at the 25 kDa level had 5.4x higher relative density than the corresponding band in NT-1 lane as well as a 22x greater band density compared to the NT-3 lane. A second high density band was observed in the NT-2 group at approximately 110 kDa, possibly a result of the accumulation of polyubiquitinated TFAM after transient inhibition of proteasomal proteolysis by MG-132 during the first two hours after SCNT and electroactivation. (Figure 13A). This TFAM pattern reverted to more uniform band density observed between individual treatments at the blastocyst stage (Figure 13B). These data reveal that the choice of protocol used for SCNT influences the accumulation of TFAM protein in the reconstructed zygotes, which could in turn affect their survival and developmental potential.

Discussion

The present data reveal an intriguing complementarity of TFAM protein distribution in male and female gametes which may reflect the complementarity organelle inheritance in the mammalian zygote. In mammals, mitochondria and mitochondrial genes are thought to be inherited clonally from the oocyte, while paternal, sperm-borne mitochondria are degraded by a substrate-specific organelle degradation process (Aoyama et al., 2006; Appleyard et al., 2006; Hiendleder, 2007; Sutovsky et al., 2003a; Sutovsky et al., 1999). Posttranslational modifications of TFAM protein such as ubiquitination, and/or TFAM segregation from sperm mitochondria into the principal piece could be conducive to maternal mitochondrial inheritance by further reducing the potential of paternal mitochondria to survive in the zygote and replicate their mtDNA.

In accordance with the above thesis, boar elongating spermatids display distinct patterns of TFAM protein accumulation in the cytoplasmic lobes. The cytoplasmic lobe is the site of translation of many proteins contributing to the formation of sperm accessory structures such as perinuclear theca, mitochondrial sheath, and fibrous sheath (Kierszenbaum, 2002; Miranda-Vizuete et al., 2003; Oko, 1998; Yu et al., 2002). However, since no TFAM labeling was observed in the mitochondrial sheath during late spermatid-stages, this suggests that TFAM may have a function other than mtDNA replication or transcription. Additionally, when inspecting ejaculated, fully differentiated boar spermatozoa the localization of TFAM was found in the principal piece of the sperm tail instead of the anticipated midpiece/mitochondrial sheath localization. Other proteins such as thioredoxins are also thought to be released from the principal piece and

translocated to the FS during spermatogenesis (Miranda-Vizueté et al., 2003). Tsga10, a protein localized in the sperm tail fibrous sheath has been shown to be expressed in the brain of adult mice (Behnam et al., 2006). The mitochondria in the midpiece of the sperm tail are thought to be responsible for the generation of ATP necessary for respiration and sperm motility. In the midpiece, the mitochondrial sheath wraps around the outer dense fibers and microtubules of the sperm axoneme (Cao et al., 2006; Kamp et al., 2003; Westhoff and Kamp, 1997). As a carrier of the paternal mitochondrial genome, the mitochondrial sheath is the expected location of TFAM protein (Sutovsky and Schatten, 2000). The mitochondrial sheath does not extend to the principal piece of the sperm tail, covered by the fibrous sheath (FS) (Cao et al., 2006; Escalier, 2006; Kim et al., 2007). The localization of TFAM to this non-mitochondrial region may suggest an alternate function of TFAM. In the human and in the rat, a smaller isoform of TFAM has been reported to be generated by alternative splicing mechanism of exon 5 ($\Delta 5$ isoform). Several pseudogenes have also been reported, in addition to the active copy of the *Tfam* gene (D'Errico et al., 2005). TFAM has also been suggested to play a critical role in maintaining mtDNA as a main component of the mitochondrial nucleoid (Kang et al., 2007). Additionally the *Tfam* gene encodes different protein isoforms targeted for mitochondria or the nucleus, perhaps of importance for regulation of nuclear and mitochondrial genomes during the process of mitochondrial biogenesis. It is possible that TFAM protein targeted towards sperm tail principal piece is a product of alternative splicing. The *Tfam* gene is widely expressed in the testis and testicular *Tfam* transcripts are alternatively spliced. One of the three testis-specific *Tfam* transcripts encodes a 26 kDa TFAM protein lacking a mitochondrial targeting signal. It is found in the nucleus of

spermatocytes and elongating spermatids in the mouse (Larsson et al., 1997a). Antibodies used in our studies did not recognize nuclear localization of TFAM on boar testicular tissue sections. Altogether, it is possible that the segregation of TFAM protein from sperm mitochondria lowers the likelihood of unwanted survival and replication of paternal mitochondria and mitochondrial genes after fertilization.

To ascertain the localization of sperm TFAM, further localization studies were conducted in the isolated sperm head and sperm tail fractions, and compared to whole spermatozoa. Some such experiments only revealed TFAM in the principal pieces of sperm tails, as shown for whole sperm preparations. However, in other experiments the sperm tails and sperm heads both displayed TFAM protein. The tail portion had principal piece labeling consistent with patterns observed in whole boar spermatozoa as predicted. Whenever observed, sperm head labeling was restricted to the postacrosomal sheath and appeared to be associated with the surface of non-permeabilized sperm heads. Western blotting experiments with isolated sperm head/tail protein extracts showed the appropriate TFAM band in the sperm tail fraction in some experiments, and in the sperm head and tail fractions in other experiments. Such discrepancies could be attributed to several variables in the preparation of sperm head and tail fractions. The process of sonication may have released TFAM protein from the sperm tails. The solubilized TFAM protein could subsequently bind to the perinuclear theca of the postacrosomal region in which the positively charged proteins including histones (Hansen et al., 2006; Kitamura et al., 2004; Tovich and Oko, 2003; Tovich et al., 2004; Westbrook et al., 2006) could be exposed by sonication-induced damage to the postacrosomal plasma membrane. Consequently, the immunolocalization and biochemical detection of TFAM

in the postacrosomal sheath (PAS) of the isolated sperm heads in some of our experiments could be due to non-specific binding of TFAM molecules released from the sonicated sperm tails. This binding of sperm tail-derived TFAM protein to the sperm heads could have occurred during separation of heads and tails in ultra centrifugation in the sucrose gradient. The release of the TFAM protein is consistent with the presence of low molecular weight anti-TFAM reactive bands in such fractions, observed despite the inclusion of appropriate protease inhibitors in the isolation buffers. It is not likely that the labeling of the PAS in the isolated sperm heads is due to non-specific antibody binding. The antibody used was affinity purified, raised against a TFAM peptide corresponding to a unique immunogenic domain not overlapping with other related proteins in the existing databases. The appropriate negative control experiment did not reveal PAS labeling. It remains to be examined if the TFAM species detected in boar spermatozoa is a testis-specific isoform or a somatic isoform (Larsson et al., 1997b).

Ubiquitination of sperm mitochondrial membrane protein prohibitin has been put forward as one possible reason for rapid proteolytic degradation of sperm mitochondria after fertilization (Thompson et al., 2003). Similarly, ubiquitination of sperm TFAM could contribute to proteasomal degradation after fertilization, and/or render sperm TFAM inactive or unable to participate in sperm mtDNA replication inside the zygote. Posttranslational modification of TFAM by ubiquitination could assist with degradation of the sperm tail components including mitochondria and mtDNA, known to occur in the mammalian zygotes (Aoyama et al., 2006; Birky, 2001; Cogswell et al., 2006; Diez-Sanchez et al., 2003a; Hecht et al., 1984; Jansen and de Boer, 1998). To date, only one convincing case of paternal mtDNA inheritance has been reported in humans, and is

associated with a mitochondrial myopathy due to a 2-bp mtDNA deletion in the *MTND2* gene (Nagy et al., 2003; Schwartz and Vissing, 2002; Schwartz and Vissing, 2003). It is possible that a targeted destruction of the sperm tail including the mitochondrial sheath after fertilization (Sutovsky et al., 1996) assures that mtDNA that could be damaged during sperm maturation and transport in the female reproductive system is not passed on to progeny (Ankel-Simons and Cummins, 1996). Substrate-specific ubiquitination of sperm mitochondrial proteins and their post-fertilization proteolysis by ooplasmic proteasomes is a plausible explanation of such a phenomenon (Sutovsky et al., 2003a; Sutovsky et al., 1999; Sutovsky et al., 2003b).

To test the hypothesis that sperm TFAM is ubiquitinated, a pull-down experiment with matrix-immobilized ubiquitin-binding UBA domain of protein p62 was performed (Kuusisto et al., 2001; Vadlamudi et al., 1996). Unconjugated ubiquitin is a 76 aa residue protein of 8.5 kDa (Ciechanover et al., 1980). To render a substrate protein recognizable to 26S proteasome, monoubiquitin first binds covalently to an internal Lys-residue of the substrate through the ubiquitin C-terminal Gly-76 residue. Subsequently, additional ubiquitin molecules bind covalently to the internal Lys-residues of substrate-bound ubiquitin in a tandem fashion. Formation of a multi-ubiquitin chain of at least four molecules (tetra-ubiquitination) adds at least 34 kDa (4 x 8.5 kDa of single ubiquitin molecule) to a target substrate protein and is a consensus signal for ubiquitinated substrate degradation by the 26S proteasome (Tomlinson et al., 2007). The 26S proteasome is composed of the 19S regulatory complex responsible for multi-ubiquitin chain recognition, deubiquitination and substrate priming, and the 20S proteasomal core in which the actual proteolysis occurs. If TFAM (24 kDa in nascent form) is

ubiquitinated, it is expected to migrate above the expected mass on SDS-PAGE. Consequently, we would expect a tetraubiquitinated TFAM species to migrate around 58 kDa, with possible ubiquitination intermediates migrating below 58 kDa and additional, polyubiquitinated species (i.e. chains of >4 ubiquitin molecules linked to 24 kDa TFAM) migrating above the 58 kDa marker. Altogether, ubiquitinated substrates form a ladder of bands on SDS-PAGE above the nascent substrate protein band, separated more or less regularly by the 8.5 kDa mass of ubiquitin. As anticipated, an anti-TFAM western blotting of ubiquitinated protein fraction affinity purified from boar sperm extracts with p62 beads revealed a prominent band at approximately 58 kDa, consistent with tetraubiquitination of TFAM (24 kDa TFAM + 4x8.5 kDa tetra-ubiquitin). A ladder effect was observed with less prominent bands above and below 58 kDa. It is not likely that such a pattern was due to dimerization/polymerization of TFAM molecules in extracts. The expected mass of multimeric TFAM would be in intervals of 24 kDa, such as 48/72/96 kDa, but no prominent bands were observed at those molecular weights. The putative ubiquitinated TFAM bands migrating above 24 kDa marker in SDS-PAGE were prominent in the p62-purified sperm extracts compared to non-purified whole sperm extracts in which the nascent 24 kDa TFAM band was predominant. The p62 pull-down method is a reliable method for identification of ubiquitinated proteins. The *p62* gene is a fast-acting early response gene involved in proliferation and differentiation, encoding a multi-domain protein with a functional role for signaling cascades for cell-surface receptors participating in protein scaffolding and linked to nuclear factor- κ B activation by I κ B ubiquitination (Rolland et al., 2007). This has a principal function for cell survival signaling involving proliferation, differentiation, and induction of anti-apoptotic genes

(Rolland et al., 2007). Although sperm TFAM may be ubiquitinated, the significance of TFAM localization in the principal piece apart from segregation from mitochondria is unclear. Further experiments need to be conducted to further assess possible functions of TFAM in the sperm tail since both ubiquitinated and non-ubiquitinated species are present. TFAM accumulation in the principal piece or its failure to translocate from the mitochondrial sheath into the principal piece during spermiogenesis could be indicative of damaged spermatozoa. Indeed, we have observed morphologically abnormal spermatozoa with TFAM labeling in mitochondrial sheath in semen of infertile men (data not shown). Experiments will be conducted to determine if higher accumulation of TFAM or its retention in the sperm mitochondria are associated with human male infertility. Double-labeling experiments with anti-TFAM and anti-ubiquitin antibodies could be conducted to confirm the link between TFAM-retention and infertility, since increased sperm ubiquitin content is observed in both infertile men (Ozanon et al., 2005; Sutovsky et al., 2001) and fertile boars producing below-average litter sizes (Lovercamp et al., 2007). Additionally, experiments addressing the fate of sperm tail TFAM after fertilization are envisioned.

According to our biochemical data, TFAM protein accumulates in porcine oocytes during meiotic maturation, and the relative content of maternally-stored TFAM is drastically reduced after fertilization. This maternal TFAM degradation after fertilization is not mimicked by artificial oocyte activation procedures used to induce parthenogenetic development or to induce embryo development after SCNT. Mitochondria are abundant in the mammalian oocyte and undergo distinct localization and structure changes after fertilization. The oocyte and early embryo are believed to have low respiratory activity

(Van Blerkom, 2004). In the mouse, the mitochondria of the oocyte and the early embryo appear underdeveloped, perhaps limiting oxidative phosphorylation or to initiate apoptosis (Dumollard et al., 2004). Changes in the mitochondrial fine structure after fertilization occur in a stage-specific manner and are consistent with higher respiratory activity required for blastomere formation. Morphological changes in the early embryo warrant a greater demand for ATP that is necessary for supporting stage-specific biosynthetic activities, plasma membrane production, formation of a fluid-filled blastocoel, and the hatching of the embryo (Van Blerkom, 2004).

In the immature porcine oocyte, mitochondrial morphology is similar to other domestic species, lacking typical somatic cell-like cristae (Krause et al., 1992). During germinal vesicle breakdown, the mitochondria translocate from the oocyte cortex to a deeper area in correspondence to the functional shift from nutrient processing of cumulus cells to providing ATP to assist cytoplasmic and nuclear maturation (Cran, 1985; Sun et al., 2001). The mitochondria of meiotically mature, metaphase-II oocytes are spherical with an electron-dense matrix with few transverse cristae (Kim et al., 1998). In the oocyte, zygotes and 2-cell and 4-cell embryos, mitochondria of various shapes and configurations are preferentially localized in to the cortical cytoplasm. As the embryo's cell number increases, the mitochondria become more dispersed. Up to the 8-cell stage, mitochondria are in aggregates enveloped by the smooth endoplasmic reticulum. This is not observed in trophoblast cells or the inner cell mass (Krause et al., 1992). Some of the mitochondria within the aggregates are linked by bridges and an amorphous mass referred to as the cytoplasmic matrix. Mitochondrial clusters in later stages of embryo development may be clustered around amorphous and/or fibrillar material. The zygotic

mitochondria are dumb-bell shaped, hooded or cup-shaped, in configuration. The mitochondrial matrix has few circular, rather than transversal cristae and is electron dense. The mitochondrial aggregates are not seen in the blastocyst (Krause et al., 1992). Also in the blastocyst the majority of the mitochondria are elongated and dispersed through the cytoplasm with the majority containing transverse cristae as found in somatic cell mitochondria. Some of these mitochondria appear to be in transition from immature (hooded) to mature (somatic cell mitochondrion-like) mitochondrial morphology, displaying lesser electron density with a content of filamentous substance, vacuoles, and aggregates of electron-dense matrix. The mitochondria in both the trophoblast and inner cell mass are epithelial-like with a less electron-dense mitochondrial matrix and more cristae (Krause et al., 1992).

The mitochondria can remain in a perinuclear clustered-pattern throughout embryo development which is suggested to be necessary for normal embryo development (Bavister, 2006). In hamsters, mitochondria were homogenously distributed in unfertilized oviductal and follicular oocytes (Barnett et al., 1996). Perinuclear aggregation of mitochondria has also been shown around the pronuclei of fertilized oocytes and around the nuclei of cleavage-stage embryos in the monkey, hamster, mouse, human (Barnett et al., 1996; Bavister and Squirrell, 2000; Nagai et al., 2004; Wilding et al., 2001). Mitochondrial clustering around the chromatin has also been observed during oocyte maturation in the cow, mouse, and pig and likely reflects the increased need for energy in form of mitochondrion-produced ATP to fuel the nuclear events during oocyte maturation and preimplantation embryo development. Accordingly, mitochondrial numbers increase during oocyte maturation in direct proportion to increased copy number

of mtDNA (El Shourbagy et al., 2006; Spikings et al., 2006; Sun et al., 2001) and the ATP concentration in the oocyte correlates with higher blastocyst rates in bovine embryos and greater *in vitro* fertilization success in humans (Barritt et al., 2002; May-Panloup et al., 2006; Reynier et al., 2001; Spikings et al., 2007). The mtDNA copy number is dependent on replication factors and transcription factors, such as TFAM (Spikings et al., 2007). This is consistent with our finding that GV-stage porcine oocytes exhibit less TFAM accumulation than metaphase II-staged oocytes. The mtDNA encodes subunits of the electron transfer chain that are responsible for cellular ATP production. Other encoded subunits and associated factors for mtDNA replication, transcription, and translation are nuclear encoded and necessary for accurate intergenomic (nuclear-mitochondrial genome) communication. As the oocyte matures, more mtDNA copies are generated, but the mtDNA copy number does not increase further until the blastocyst stage. This is consistent with our observation that the maternal pool of TFAM protein is reduced by proteolysis after fertilization. Contrary to our findings at protein level, there appears to be a near-linear increase in TFAM mRNA content between the GV oocyte and blastocyst stage *in vivo*. An increase in TFAM mRNA levels is observed at the 4-cell stage *in vitro* in porcine embryos compared to mRNA levels in early preimplantation development (Spikings et al., 2007), which is consistent with our data. It is possible that the increase in TFAM mRNA prior to blastocyst formation may serve to generate a pool of mRNA to be translated after blastocyst hatching and implantation, when rapid proliferation occurs in both trophoctoderm and the embryo proper. A strict regulation of mtDNA copy number by mtDNA replication is vital for embryo development and

differentiation of the normal preimplantation embryo (Bowles et al., 2007; Kameyama et al., 2007; Spikings et al., 2007; St John et al., 2005b; Thundathil et al., 2005).

Maternal transmission of mitochondria and mtDNA, termed homoplasmy is the norm in mammals (Birky, 1995; Bowles et al., 2007; Kuroiwa, 1991). Approximately 100,000 copies of maternal mtDNA are contributed to the embryo (Shoubridge, 2000) and this pool must be sufficient to sustain the mitochondrial population in the dividing embryo until the blastocyst stage. Therefore, it is necessary that the fertilization-competent metaphase-II oocyte will have sufficient mtDNA content and complement of mitochondria to full embryo development and absorb mitochondrial dilution (progressively reduced number of mitochondria per blastomere) during rapid proliferation (Sun et al., 2007). Less mtDNA replication could lead to a progressive decrease in mtDNA content in cleaving embryos (Spikings et al., 2007).

The labeling of TFAM protein in pronuclear zygotes is consistent with labeling of TFAM in metaphase-II oocytes. A punctate pattern of TFAM labeling was present within the cytoplasm of the blastomeres at day 7. No obvious differences were observed between the staining pattern of trophectoderm and inner cell mass cells. Blastomeres of the blastocyst stage porcine embryos appear to have a relatively low content of mtDNA possibly due to a low level of mtDNA replication during preimplantation development (Spikings et al., 2007). Consistent with this observation we have found a low relative amount of TFAM protein in porcine zygotes at 22 hours post-fertilization. When comparing porcine zygotes produced by *in vitro* fertilization to parthenogenically activated oocytes at 22-hours after fertilization/activation by western blotting-densitometry, the relative amount of TFAM in the parthenotes was 44.6-fold higher than

in *in vitro* fertilized embryos. Replication of mtDNA is thought to occur at low levels or not at all during early embryogenesis in the mouse (Thundathil et al., 2005) and reported in pig (Spikings et al., 2007). It is well documented that chemical or electrical artificial activation of mammalian ova does not properly recapitulate early events of normal embryo development, including the activation of anti-polyspermy defense (Wang et al., 2003) or recruitment of maternally stored mRNAs and proteins (Svoboda et al., 2000). These altered early embryo development events likely affect translation of maternally stored mRNAs and the timing of maternal-embryonic transition of transcriptional control. Also it has been shown that overexpression of human TFAM in mice increases the mtDNA content in the heart in transgenic mice (Ikeuchi et al., 2005) as well as increasing cardiac dysfunctions and myocardial infarction (Kang et al., 2007). Consequently, proteins translated from maternally stored mRNA, such as TFAM may be overproduced as shown in our parthenogenetic and SCNT-reconstructed porcine zygotes. Similarly, overexpression of TFAM has been shown in cloned mouse embryos (Vassena et al., 2007). Although genetic ablation of *Tfam* gene is embryo-lethal by day e10.5 (Larsson et al., 1997a), overexpression at early stages of embryo development when a low mtDNA replicating activity is favored could have equally damaging effect on embryo development. Such an effect will be examined in further studies employing *Tfam* gene overexpression (mRNA injection into a zygote) and knock-down (RNA-interference). Among possible reasons for TFAM protein accumulation in cloned embryos and parthenogenetically activated ova is that more TFAM mRNA recruitment for translation occurs during artificial activation or that the pathway for maternal mRNA

degradation is not as functional as it would be under physiological conditions after natural fertilization.

Conclusions

The results described in the present thesis allow us to conclude that the TFAM protein displays an extra-mitochondrial localization in the boar spermatozoa and is detected in the sperm tail principal piece rather than the expected midpiece localization of the sperm mitochondrial sheath. TFAM protein is inserted in the sperm tail principal piece in the late haploid phase of spermatogenesis and is posttranslationally modified by ubiquitination. The oocyte-content of TFAM protein increases during oocyte maturation from the germinal vesicle stage to the metaphase II stage. Proteolytic degradation of maternally stored TFAM protein occurs following fertilization. Artificially activated oocytes and zygotes reconstructed by somatic cell nuclear transfer display a greater content of TFAM protein compared to *in vitro* fertilized oocytes. TFAM mRNA content increases from the GV-stage up to the blastocyst stage *in vivo* but not during *in vitro* culture of porcine embryos.

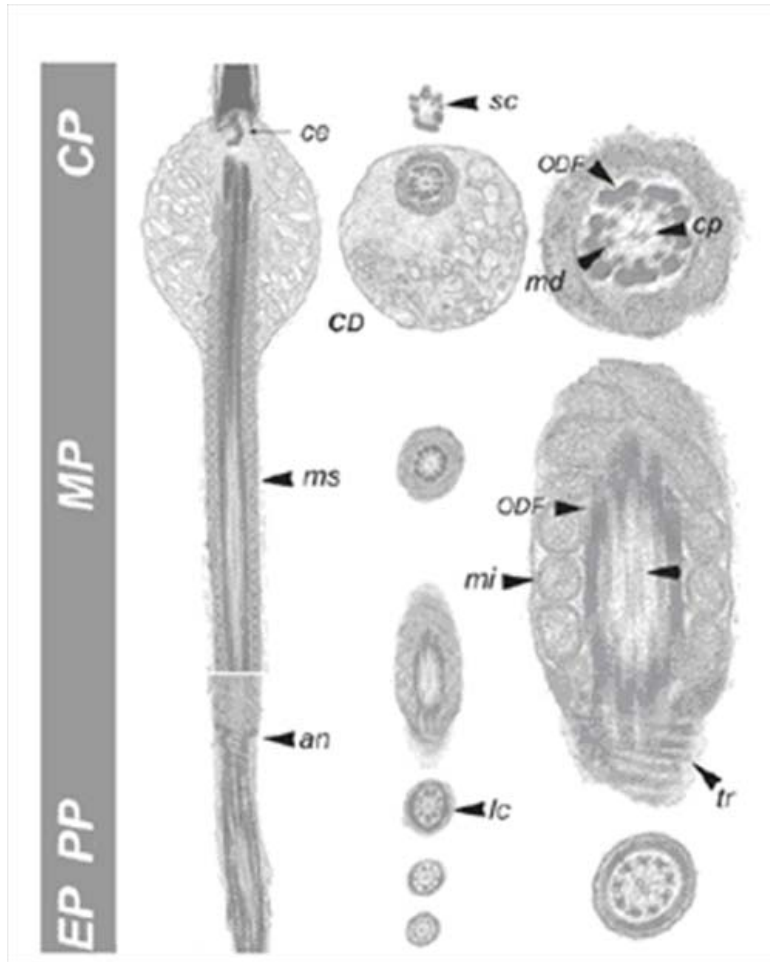


Figure 1: Ultrastructure of the mammalian sperm tail. In the connecting piece (CP), the proximal centriole (ce) is embedded in the dense structure of the sperm capitolium surrounded by the striated columns of the outer dense fibers (sc). The mitochondrial sheath (ms) wraps around the outer dense fibers (ODF) in the midpiece (MP). The principal piece (PP) is separated from the midpiece by the annulus (an). Transversal ribs (tr) of the fibrous sheath are prominent in the principal piece. The end piece (EP) is not covered by fibrous sheath. Illustration courtesy of Dr. P. Sutovsky and Dr. G. Manandhar, adapted from (Manandhar and Sutovsky, 2007).

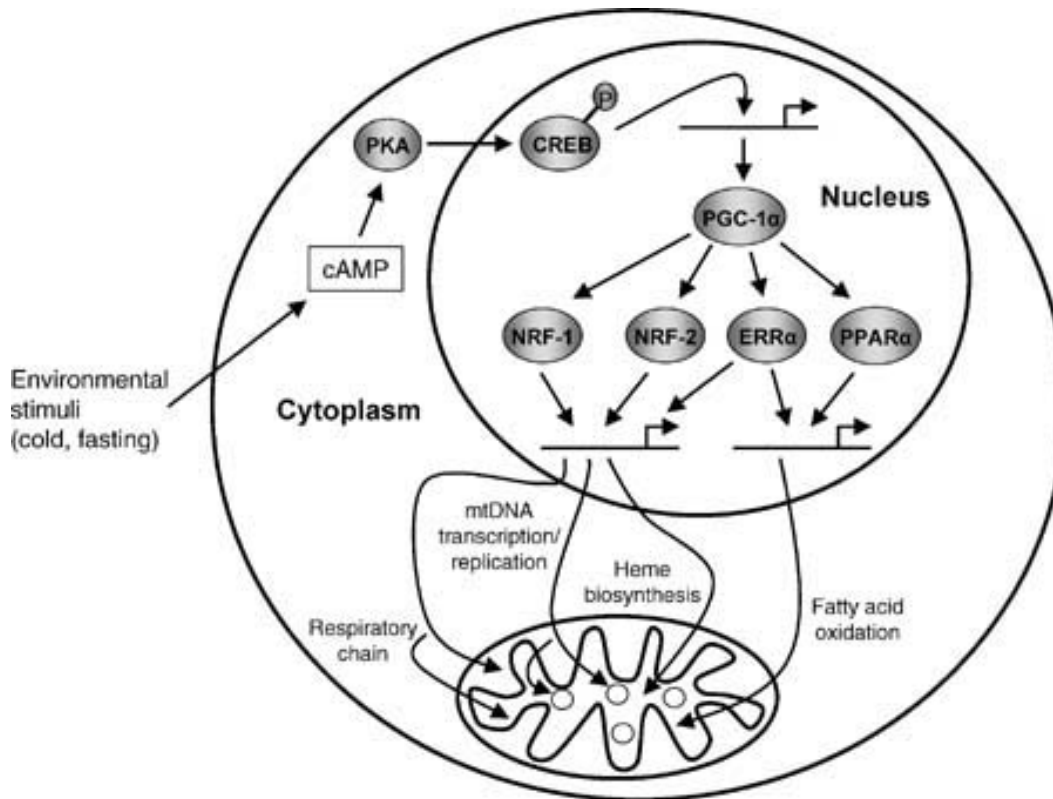
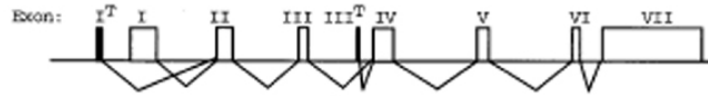


Figure 2: The expression of *Tfam* gene is co-regulated by two transcriptional activators, PGC-1 α and NRF-1. Environmental stimuli such as cold, fasting, or increased oxygen uptake trigger the cAMP-dependent signaling from CREB family of transcription factors which then causes the translation of the nuclear encoded protein PGC-1 α that directly increases translation of NRF-1 protein which assists in shuttling TFAM protein into the mitochondrial matrix. Adapted from (Scarpulla, 2006).

A



B

Splice donor sites

Consensus	A	g
Consensus	CAGgtaagt	
Exon I	CGTCTATCAGgtaggccgcg	Intron I
II	AAACACCAGgtagggaggt	II
III	AGAAAAAGgtaagcggttc	III
IV	AAACAGAGAGtagatca	IV
V	TTCGCTCAGgtagcagag	V
VI	GGAAAAAGAGtaggtatta	VI
I ^T	GGCCTGGGGgtagccgcc	
III ^T	GTCCACACAGgtaattcttc	

Splice acceptor sites

Consensus	- t/c >11 - ncagS	t
Intron I	ttctttttacttgtgtgtagTCTGTCTGT	Exon II
II	ttaatccattctattttagATGCAAACT	III
III	attgaggttttccacacagTPTATGAAG	IV
IV	cactaatttttgttactcagGATTAATTT	V
V	ttaactatcttgttctacagGGAAATTTGA	VI
VI	attgtcatgttctttctcagGCATATATTC	VII

C

```

Tfam (m-mTFA)  -129 ggctccgcccccctctcctcgggccggcccccgccactgagcgggtgggggagc -80
                |||||  |||||  ||  ||  |||||  |||||  |||||  |||||
TFAM (h-mTFA) -117 ggctcttattctctcccccggagg..ccgccacc.....ggggtacg -77
                -79 cacagcactcctgt.....cctcccgcctcctcgcgcggac -42
                ||  ||  ||  |||||  ||  |||||  ||  ||  |||||
                -76 ctctcccgcctcgcgcaattccgcccggcccccacatctaccggac -27
                   NRF-1                      Sp1                      NRF-2
                   +1
                -41 cggaaagt..cgggcttcccacagtaaccccggcgcgggggcATGATAA 7
                   |||||  ||  ||  |||||  |||||  |||||  |||||  |||||
                -26 cggatctttagcagatttcccatagtgCCTCGTAGTGGGGGCGATGATAA 24
                   NRF-2                      +1
                8  CAAGCCCCCGAGTTCACAGCTGGT 33
                   ||  ||  ||  ||  |||||  |||||
                25 CACAGCCCGAGGGTCACAGCGGGT 50
    
```

Figure 3: Structure and nucleotide sequence for murine *Tfam* gene. A. *Tfam* gene structure. Exons I-VII are widely expressed (open boxes); testis expressed exons are I^T and III^T (filled boxes). B. Splice acceptor and donor sites for *Tfam* gene. C. Promoter region sequence alignments of *Tfam* and *TFAM* genes. Sp1, NRF-1, and NRF-2 binding sites are shown. Adapted from (Larsson et al., 1997a).

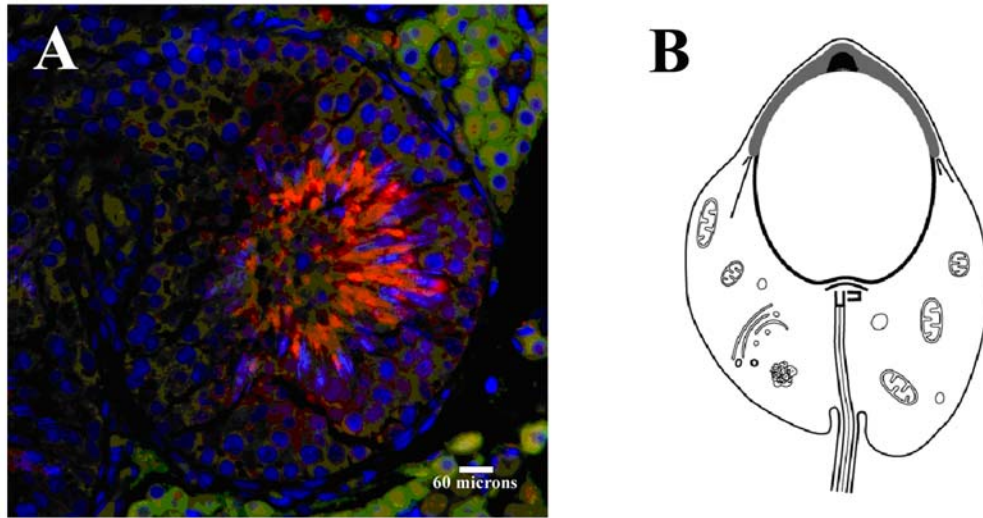


Figure 4: Distribution of TFAM (red) during spermatogenesis in boar. A. Testicular tissue section displaying TFAM accumulation in the cytoplasmic lobes of elongating spermatids, possibly as a result of TFAM protein translation in the lobe. No distinct TFAM labeling was associated with the developing mitochondrial sheath, which is already present at this step of spermiogenesis, suggesting a segregation of TFAM from spermatid mitochondria. B. Diagram of elongated spermatid depicting mitochondrial localization within the cytoplasmic lobe. Illustration courtesy of Dr. G. Manandhar, adapted from (Manandhar and Sutovsky, 2007).

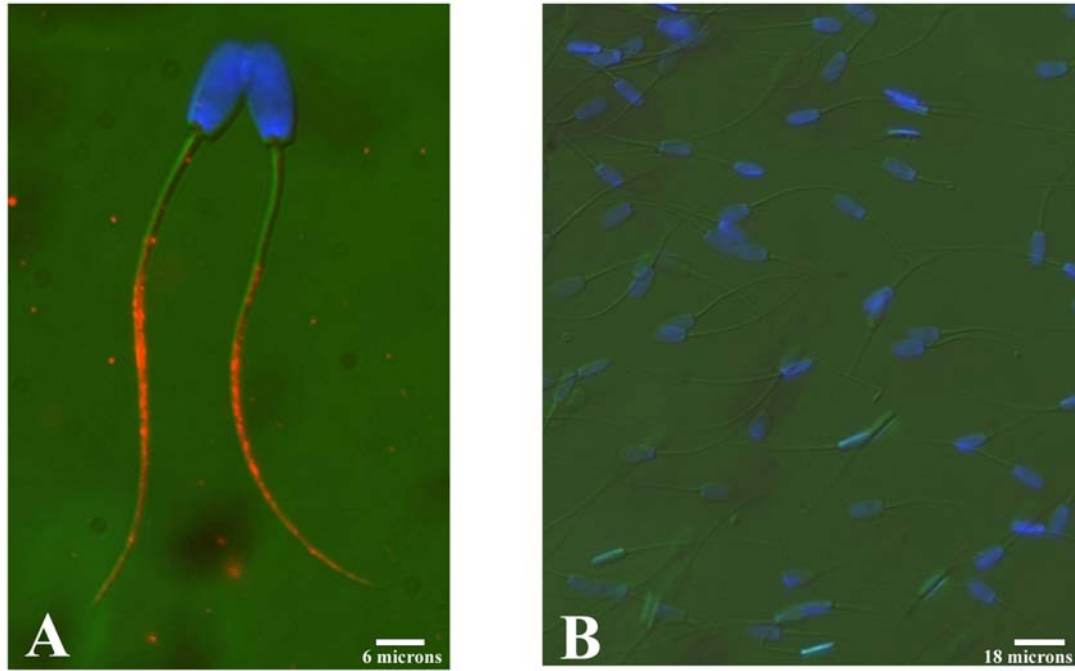


Figure 5: Immunofluorescence labeling of TFAM protein in ejaculated boar spermatozoa. A. TFAM labeling (red) is located in the sperm tail principal piece of mature, ejaculated spermatozoa. B. Negative control labeling with non-immune rabbit serum.

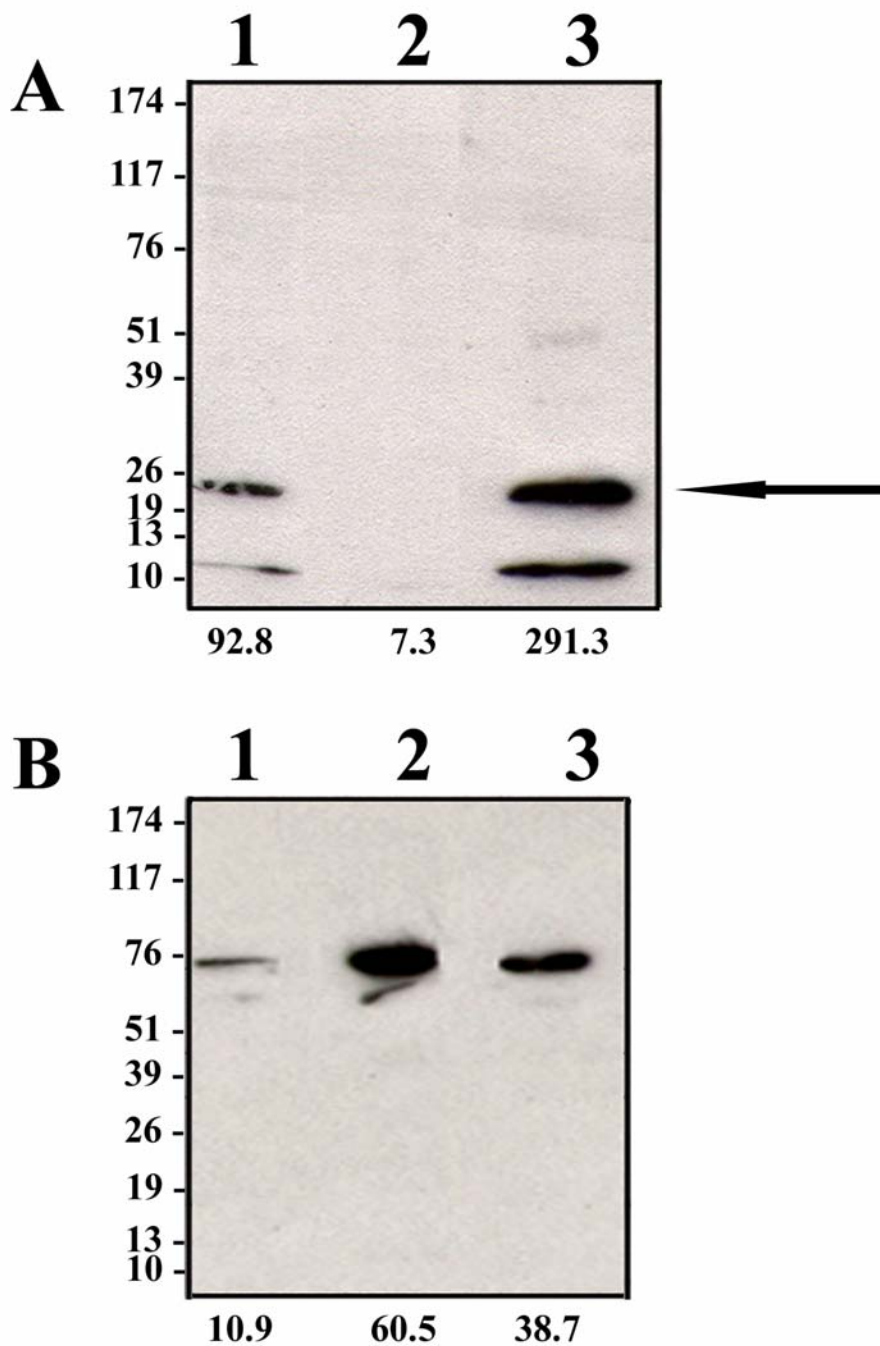


Figure 6: Western blotting of TFAM (A) and β -actin (B; loading control) in boar sperm extracts. A. A major TFAM band of approximately 25 kDa is present in the whole sperm extract (lane 1) and sperm tail (lane 3), but not in the sperm head extracts (lane 2). B. Loading control with anti-beta-actin antibody, also with sperm extracts of whole spermatozoa (lane 1), sperm heads (lane 2), and sperm tails (lane 3). Relative band densities were calculated by sum intensity values as listed numerically below each lane. All listed values are to be multiplied by 1×10^3 for corrected optical intensity.

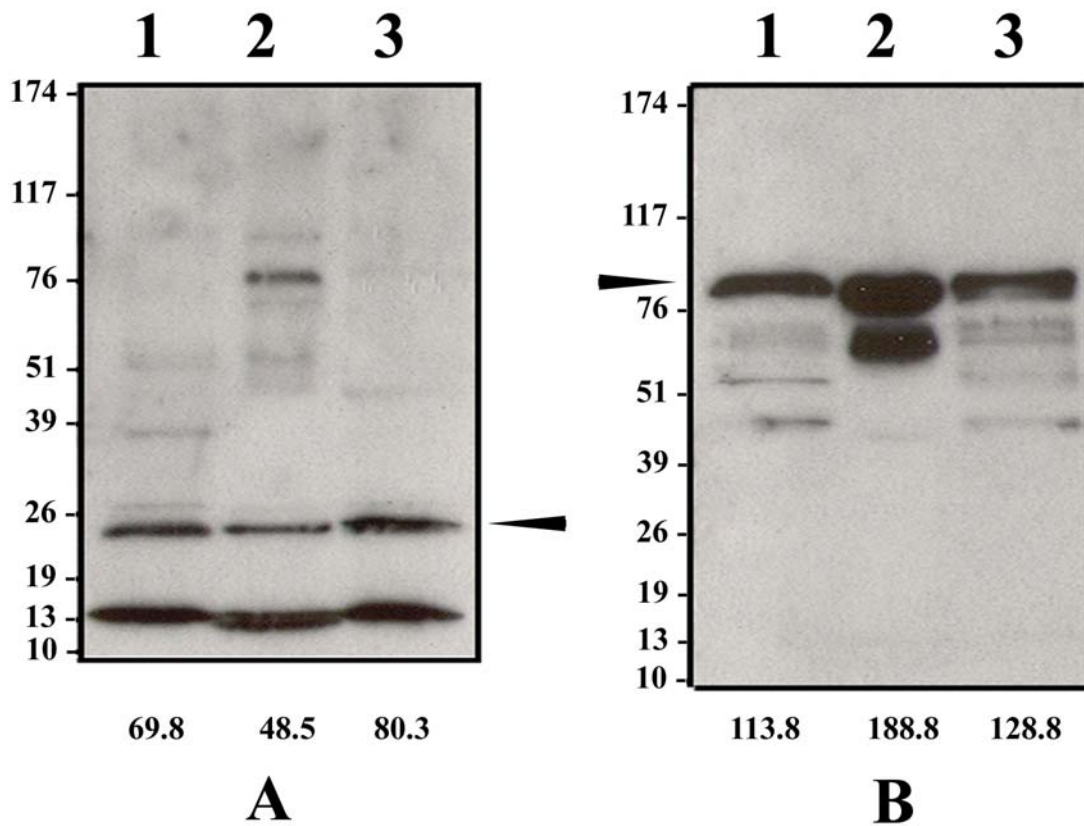


Figure 7: Western blotting of TFAM (A) and β -actin control (B) in boar sperm head and tail extracts illustrates TFAM degradation products (13 kDa band) and binding of solubilized TFAM protein to isolated sperm heads after prolonged sperm sonication. A. TFAM accumulation at approximately 25 kDa and 13 kDa is shown in whole sperm (lane 1), sperm head (lane 2), and sperm tail (lane 3) extracts. B. Control beta-actin band is reflective of protein loading of whole sperm (lane 1), sperm head (lane 2), and sperm tail (lane 3) extracts. Relative band densities were calculated as the sum intensity values and listed numerically below each lane. All listed values are to be multiplied by $1 * 10^3$ for corrected optical intensity.

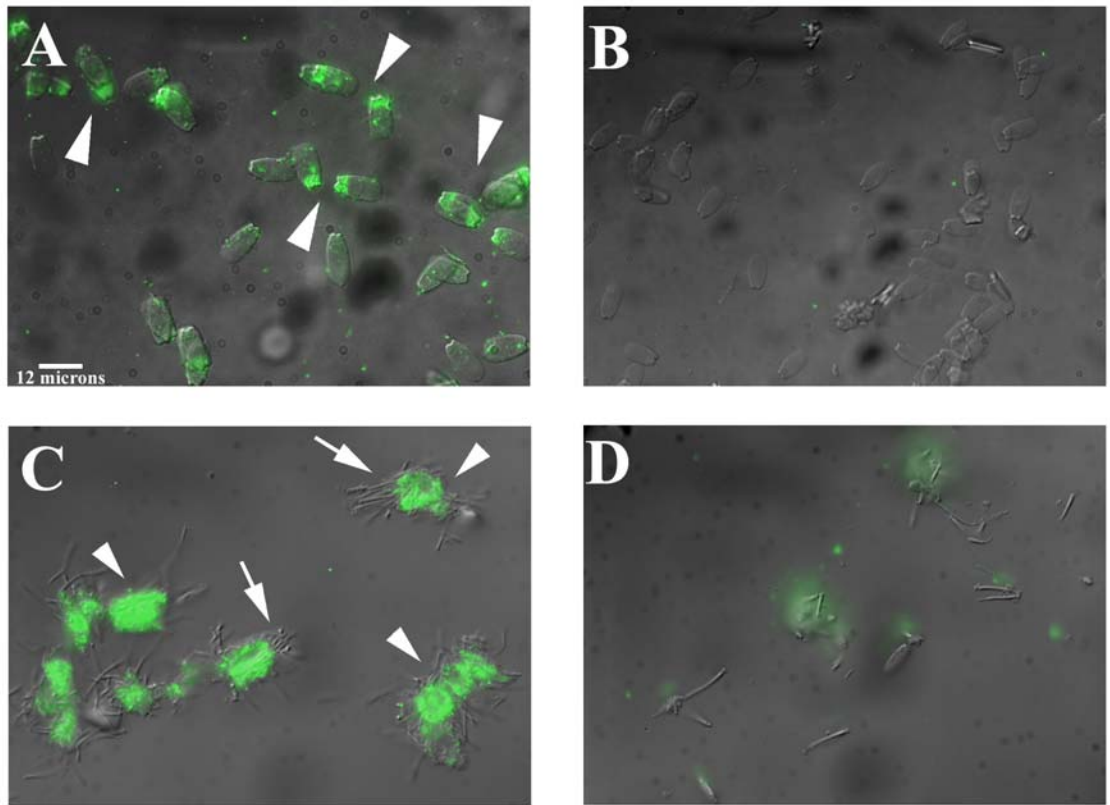


Figure 8: Anti-TFAM (A, C; green) and normal rabbit serum (NRS; negative control) labeling in sonicated boar sperm head (A, B) and tail (C, D) preparations. A. Inconsistent labeling of TFAM protein (green; arrowheads) in the post-acrosomal sheath of isolated sperm heads, likely resulting from TFAM protein release from the sperm tail during prolonged sonication. B. Normal rabbit serum control shows no specific labeling in sperm head preparation. C. Labeling of TFAM protein (green) in the principal pieces of the isolated sperm tail fraction. Note that no labeling is seen in the midpiece portions of the isolated tails (arrows). D. Normal rabbit serum control lacks specific labeling the sperm tail preparation.

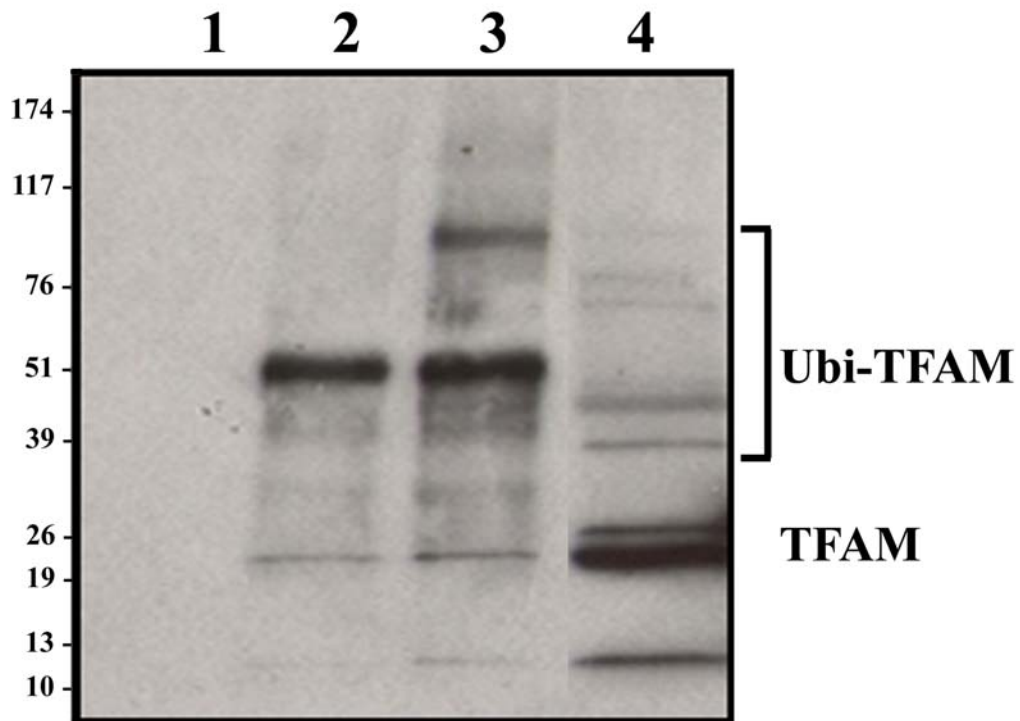


Figure 9: Affinity purification of ubiquitinated proteins and detection of ubiquitinated TFAM-species in boar spermatozoa. Sperm extracts were incubated with synthetic ubiquitin-binding UBA-domain of ubiquitin-interacting protein p62, immobilized on agarose beads. Affinity purified ubiquitinated protein fraction was probed by western blotting with anti-TFAM antibody. Control p62 fraction (no sperm extract exposure) shows no labeling (Lane 1). After coincubation with boar sperm extracts, p62 lane shows only a minor residual band at the 25 kDa, corresponding to non-ubiquitinated TFAM, while the larger, ubiquitinated TFAM-bands are dominant (Lane 2). Additional high mass TFAM bands were revealed by doubling of the protein load of p62-purified proteins (Lane 3). In absence of p62-assisted purification, the non-ubiquitinated, nascent 25 kDa TFAM band is most dominant in boar sperm extracts (Lane 4).

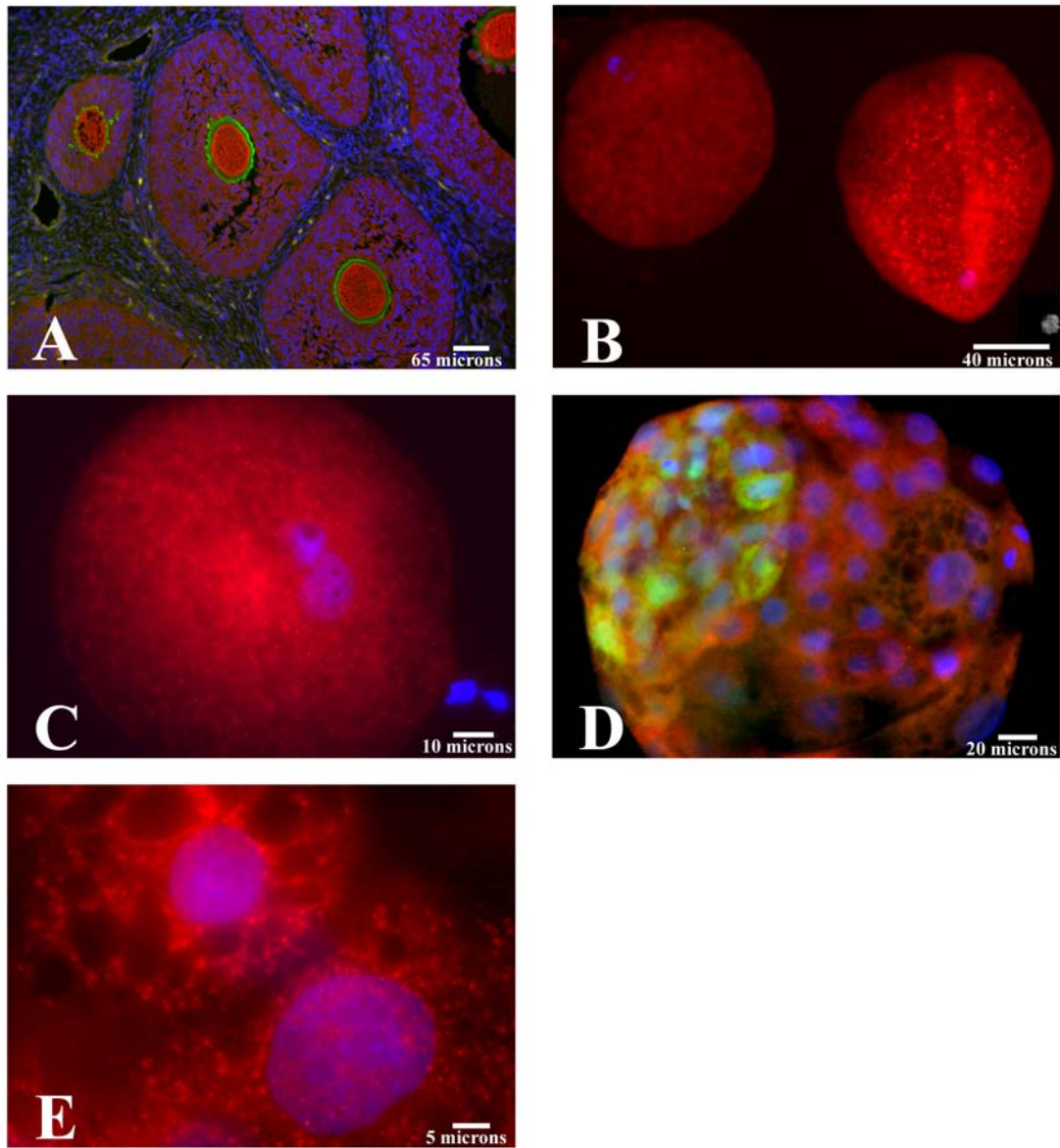


Figure 10: Immunofluorescence labeling of TFAM (red) in porcine oocytes and preimplantation embryos. Typical, representative specimens are shown. A. Accumulation of TFAM in the cytoplasm of immature porcine oocytes, as detected on tissue sections of porcine ovary. Zonae pellucidae are labeled green. B. Whole mounted GV-stage oocytes (left) show lesser TFAM accumulation/fluorescence intensity than meiotically mature MII-oocytes (right) when examined on the same microscopy slide. C. The pronuclear-stage zygote at 19 h after fertilization shows less TFAM labeling than that of MII-oocytes. D. Cytoplasmic localization of TFAM in a day 7 porcine blastocyst produced by *in vitro* fertilization and embryo culture. Green labeling represents microtubules in the inner cell mass. E. High magnification detail of individual blastomeres in the same blastocyst shows typical TFAM distribution corresponding to individual mitochondria.

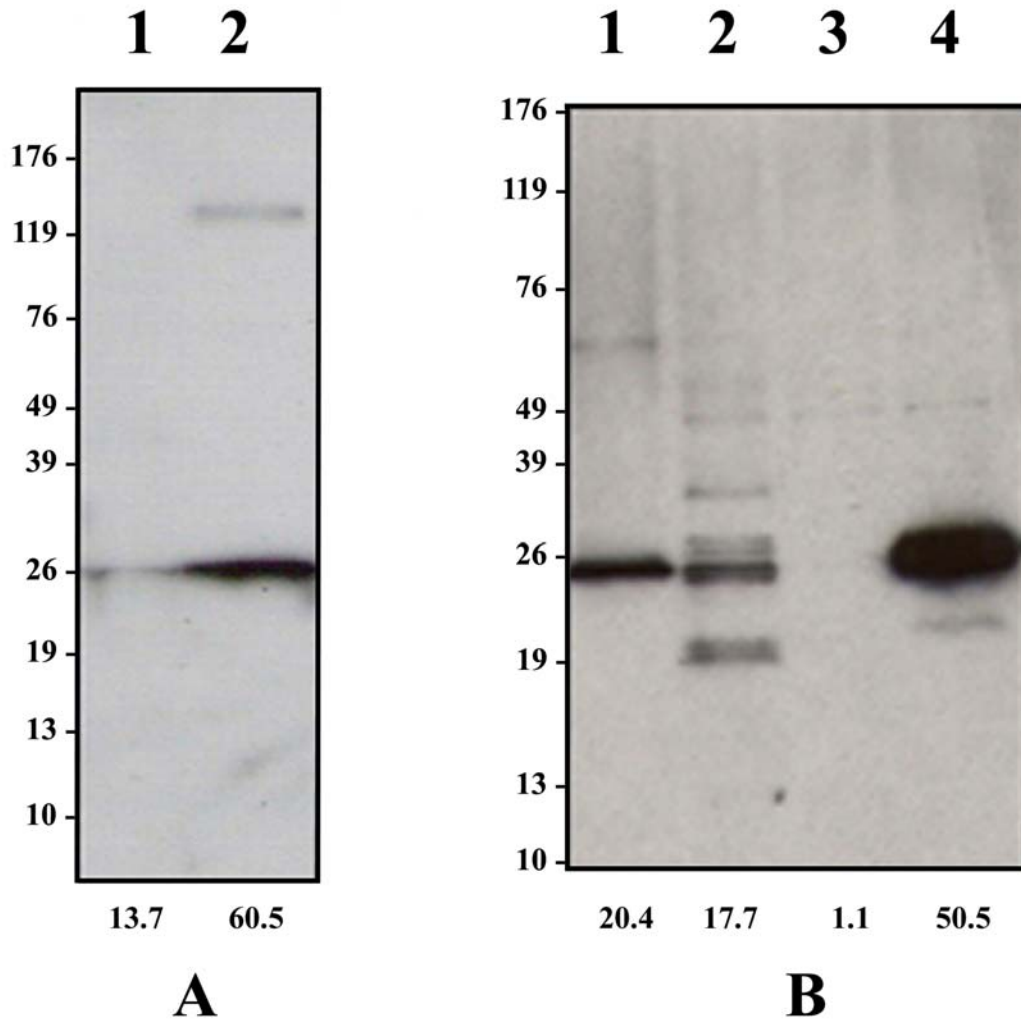


Figure 11: Western blotting of TFAM in 100 porcine oocytes/zygotes per lane, collected at various stages of meiotic maturation and development. The relative amount of TFAM is higher in MII oocytes (A, B; lane 2) compared to GV-oocytes (A, B; lane 1) or zygotes collected at 22 h post fertilization *in vitro* (B; lane 3). Parthenogenetically activated oocytes at 22 h post activation (B; lane 4) show a high content of TFAM protein compared to *in vitro* fertilized oocytes 22 h post-fertilization (B; lane 3), GV oocytes (B; lane 1), and MII oocytes (B; lane 2). Relative band densities were calculated as the sum intensity values and listed numerically below each lane. All listed values are to be multiplied by 1×10^3 for corrected optical intensity.

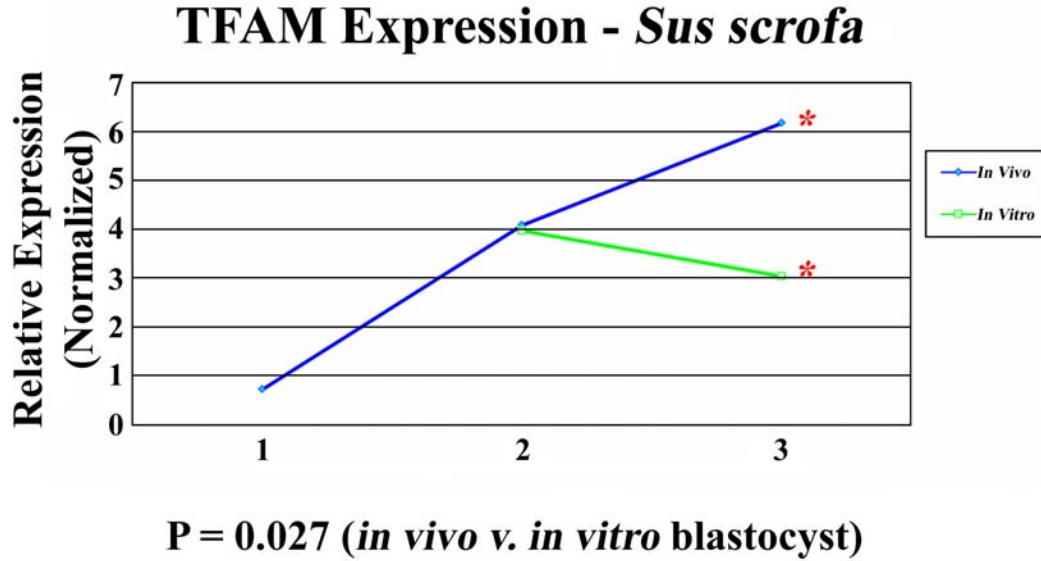


Figure 12: Relative content of TFAM mRNA expression in the porcine GV-stage oocytes (1), 4-cell embryos (2), and blastocysts (3). The relative normalized expression in *in vivo* blastocysts compared to *in vitro* blastocysts is increased between GV-stage and blastocyst *in vivo*, while there is a significantly reduced expression in the *in vitro* blastocysts compared to *in vivo* blastocyst. Data from K. Whitworth (Whitworth et al., 2005).

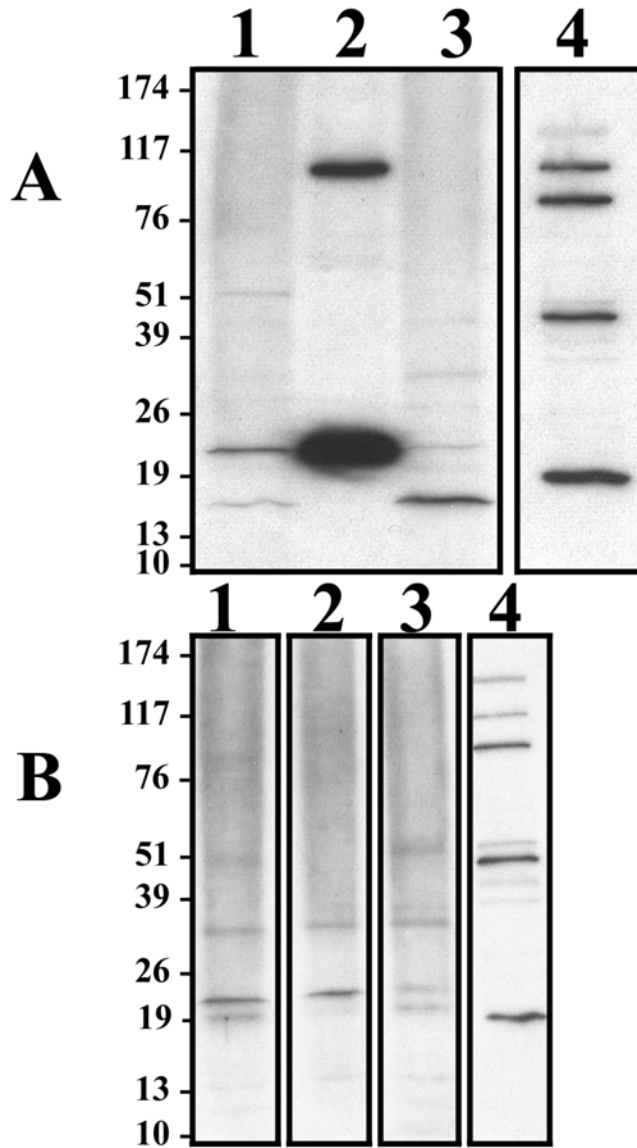


Figure 13: Western blotting of TFAM in porcine zygotes (A) and day 7 blastocysts (B) reconstructed by using three different protocols for somatic cell nuclear transfer (NT-1, NT-2, NT-3). Extracts of 27 zygotes/blastocysts were loaded per lane A. TFAM was detected by western blotting after conventional SCNT procedure after electrofusion (NT-1; lane 1), electrofusion followed by a two hour treatment with a reversible proteasomal inhibitor MG-132 (Sutovsky and Prather, 2004) (NT-2; lane 2), and after chemical activation with thimerosal (NT-3; lane 3) (Machaty et al., 1997). Relative band densities of the 25 kDa TFAM band are as follows: NT-1 83.8×10^3 , NT-2 452.7×10^3 , and NT-3 20.0×10^3 . The liver cell extract-control is also shown (Lane 4). B. The 25 kDa TFAM band shows similar densities in NT-1 and NT-2 blastocysts, but its density is reduced in NT-3 blastocysts. Relative band densities are as follows for 25 kDa: NT-1 44.6×10^3 , NT-2 33.6×10^3 , and NT-3 17.1×10^3 . Images kindly provided by Dr. G. Manandhar.

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VITA

Jennifer L. Antelman was born in Takoma Park, MD on July 11, 1982. She grew up in Clarksville, MD which lies between Baltimore and Washington, DC. She attended the University of Maryland – College Park from 2000 to 2004 and graduated with a B.S. in Animal Sciences. Throughout her undergraduate career she obtained research experience in poultry nutrition, equine nutrition, aquaculture behavior, soil and manure sciences, and molecular biology. Jennifer has also enjoyed teaching subjects such as art theory, introduction to animal science, and reproductive physiology.

She has a publication in the 19th Equine Science Society Symposium describing her work in reviewing feeding management practices of thoroughbred racehorse trainers. Jennifer has also presented at the 39th Society for the Study of Reproduction meeting in Omaha, NE in 2006, reporting on her work with mitochondrial transcription factor A (TFAM). Jennifer has received awards such as East Asia Scholarship for her work as a co-developer initiating an advanced anatomy and physiology class using problem based learning techniques incorporating veterinary acupuncture. She has received first place in the University of Missouri, Department of Animal Sciences Graduate Student Forum for her presentation on TFAM. Additionally, Jennifer was awarded a departmental travel award to attend the SSR 2006 meeting.

Jennifer plays an active role in organizations such as the Animal Science Graduate Student Association of University of Missouri, is a member of the MU

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Jennifer has a passionate interest in animal sciences as well as human biomedical sciences and hopes to pursue further education in medical studies.